CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of Pharmaceutical Chemistry and Pharmaceutical Analysis

Isolation and characterization of gangliosides recognized by *Helicobacter pylori* sialic acid binding adhesin SabA

DIPLOMA THESIS

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ERASMUS Project

September – December 2015

DECLARATION

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Hradec Králové 2016

Thuy Duong Nguyen

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ABSTRACT

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Title of diploma thesis: Isolation and characterization of gangliosides recognized by *Helicobacter pylori* sialic acid binding adhesin SabA

Nowadays, *Helicobacter pylori* infection occurs in approximately 50% of mankind. It frequently causes gastric mucosal inflammation that may further lead to gastric and peptic ulcer disease and eventually result in gastric cancer. As it represents an important risk factor for gastric malignancies, it has been classified as carcinogen class I by IARC since 1994. Nevertheless, responses to the infection vary in either severity or extent due to diverse bacterial virulence. However, adhesion to the host's gastric mucosa plays a crucial role in successful colonization, thus understanding the mechanism and the role of adhesins have a great impact on treatment strategy. Therefore, our main aim included isolation and structural characterization of gangliosides recognized by *H. pylori* adhesin SabA.

Firstly, the gangliosides needed to be isolated from total acid fraction of human liver metastasis from lung cancer. We used silica gel column chromatography and after several repurifications, we finally selected five fractions for subsequent testing. Samples were sent to structural analysis by mass spectrometry and besides that used for chromatogram binding assays with antibodies, lectins and *H. pylori* strains.

In summary, we obtained a characterized three different gangliosides that were bound to *H. pylori* strain expressing SabA. Despite strenuous efforts, we were not able to obtain pure compound thus further purifications and closer structural analysis will be perform in the future.

Keywords: *Helicobacter pylori*, gangliosides, SabA, chromatogram binding assay, mass spectrometry

ABSTRAKT

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Název diplomové práce: Izolace a charakterizace gangliosidů rozpoznávaných adheziny *Helicobacter pylori* SabA vázající kyselinu sialovou

Bakterií *Helicobacter pylori* přežívající v kyselém žaludečním prostředí je v dnešní době infikována téměř polovina populace. Infekce nejčastěji způsobuje záněty žaludeční sliznice, které mohou později vést k atrofii a rozvoji intestinální metaplazie, vředové chorobě gastroduodena a případně i způsobit rozvoj karcinomu žaludku. Odpověď organismu na přítomnost infekce se nicméně liší v závislosti na mnoha vedlejších faktorech, zejména pak na virulenci bakterie. Přilnutí k žaludeční sliznici hostitele představuje zásadní krok k úspěšné kolonizaci, tudíž objasnění mechanismu a role bakteriálních adhezinů má zásadní vliv na sestavení účinného léčebného plánu. Naším cílem tedy byla izolace a charakterizace gangliosidů, které jsou rozeznávány *H. pylori* SabA adheziny.

Prvním krokem byla izolace gangliosidů z kyselé frakce lidských jaterních metastáz pocházejících z plicního karcinomu. Čištění bylo opakovaně prováděno pomocí sloupcové chromatografie. Celkově pět frakcí bylo následně vybráno k dalšímu testování. Vzorky z těchto frakcí byly použity k vazebným testům s protilátkami, bakteriálními kmeny *H. pylori* a lektiny, současně s tím byly charakterizovány pomocí hmotnostní spektrometrie.

Ve výsledku se nám podařilo získat a charakterizovat tři různé gangliosidy, které byly rozeznány kmeny *H. pylori* disponující SabA adhesiny. Navzdory veškerému úsilí se nepodařilo získat zcela čisté sloučeniny, a proto bude další purifikace spolu s podrobnější strukturální analýzou předmětem dalšího studia.

Klíčová slova: *Helicobacter pylori*, gangliosidy, SabA, vazebné testy, hmotnostní spektrometrie

TABLE OF CONTENTS

1. LIST OF ABBREVIATIONS

AAL	*Aleuria aurantia* lectin
BabA	blood group antigen binding adhesin
CagA	cytotoxin-associated gene A
Cer	ceramide
ESI	electrospray ionization
Fuc	fucose
Gal	galactose
GalNAc	*N*-acetylgalactosamine
Glc	glucose
GlcNAc	*N*-acetylglucosamine
Hex	hexose
HexNAc	*N*-acetylhexosamine
HP-NAP	*Helicobacter pylori* neutrophil-activating protein
IARC	International Agency for Research on Cancer
LC	liquid chromatography
m/z	mass-to-charge
MS	mass spectrometry
NeuAc	*N*-acetylneuraminic acid/sialic acid
NSAID	nonsteroidal anti-inflammatory drug
PBS	phosphate buffered saline
PBS/BSA	phosphate buffered saline containing bovine serum albumin
PPI	proton pump inhibitor
VacA	vacuolating cytotoxin A

2. INTRODUCTION

2.1. General introduction to glycobiology

The origin of glycobiology was established as a coupling of carbohydrate chemistry and biochemistry. It represents a branch of science that encompasses studies of the structure, biosynthesis and biological functions of glycans. Glycans have a number of various functions, such as providing structural components, affecting intrinsic protein properties and mediating both extracellular and intracellular interactions with other molecules.

Glycoconjugates are formed of two main parts. Sugar-unit, consisting mono-, oligo- or polysaccharides, is attached to aglycone, which may be either protein or lipid. Depending on the type of linkage and non-sugar component, three major classes of glycoconjugates are commonly recognized: glycoproteins with *N*-linked or *O*-linked glycans, proteoglycans and glycosphingolipids.

N-glycans are defined by oligosaccharide, covalently bound to amide nitrogen of asparagine moiety on polypeptide chain. On the other hand, the sugar-fragments in *O*-glycans are attached to the polypeptide backbone *via* *N*-acetylgalactosamine binding to hydroxyl group of threonine or serine residue [1].

As the third class, glycosphingolipids play a more important role in this thesis, it is characterized in a next, single chapter.

2.1.1. Glycosphingolipids

Glycosphingolipids (GSLs) represent a type of amphipathic compounds that are built on ceramide lipid moiety, formed by long-chain amino alcohol sphingosine coupled *via* amide linkage to fatty acid. Hundreds of different glycans may be then attached to the lipid core chain and combined with a ceramide part varied in length, number of hydroxyl groups or multiple bonds, leading to great diversity in structure of GSLs (Fig. 1.). Despite that fact, there are still some clear and logical ways to sort them into groups.

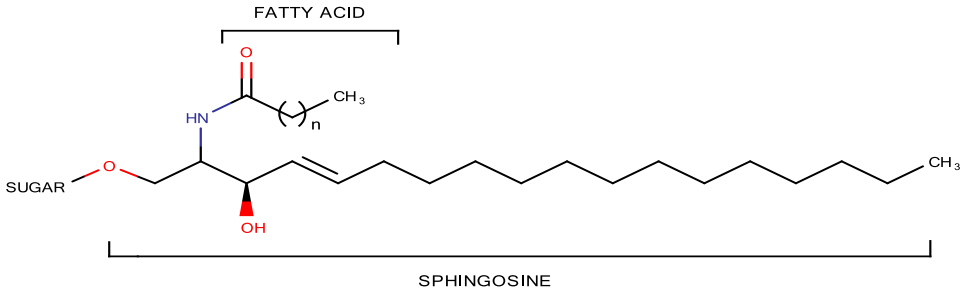

Fig. 1. Common structure of glycosphingolipids

Vertebrate GSLs are characteristic with β-linked galactose (GalCer) or glucose (GlcCer) as the first saccharide attached to the ceramide moiety. Generally, the categorization for nomenclature is based on the further extended neutral core structure (Table 1). Furthermore, each GSL subfamily shows cell- or tissue-type specificity, thus they are mainly found in certain sections of the organism. The variety correlated with different purpose can be observed, for example in mammals, where GSLs with lacto-series primarily occur in secretory organs and on the other hand, neolacto-GSLs are frequently expressed on certain hematopoietic cells. Compared to that, ganglio-subfamily is mostly found in brain, while globo-series GSLs are typical for erythrocytes.

subfamily series	**structure**	**abbreviation**
lacto	Galβ1-3GlcNAcβ1-3-	Lc
neolacto	Galβ1-4GlcNAcβ1-3-	nLc
ganglio	Galβ1-3GalNAcβ1-4-	Gg
globo	Galα1-4-Gal	Gb
isoglobo	Galα1-3-Gal	iGb

Table 1. Names and abbreviations of common core structures of vertebrate glycosphingolipids

According to another classification, glycosphingolipids can be characterized as acidic or neutral type. Non-acidic GSLs have no charged or ionic groups, whereas the rest of them are substituted with either sialic acid or sulfate. Sialylated GSLs are commonly termed as gangliosides [1].

2.1.1.1. Biosynthesis of glycosphingolipids

The entire complex process of glycosphingolipids forming begins on the cytoplasmic face of endoplasmic reticulum with the synthesis of ceramide. This component represents the hydrophobic part of the GSL consisting of a long-chain base (sphingoid) connected *via* amide linkage to a fatty acid. Resulting ceramide molecule is subsequently coupled to the first sugar residue from nucleotide sugar donors with the aid of glycosyltransferases and thereafter shifts into Golgi apparatus. Further sugar elongation of the core chain then proceeds in the Golgi lumen, eventually ended with an addition of sulfate or sialyl moiety. The final step includes the transfer of glycosphingolipids through cell lumen compartment to the plasma membrane.

2.1.1.2. Biological functions of glycosphingolipids

A number of different GSLs functions have been observed and described over the years, however in summary, they may be grouped into two main categories. As they are essentially presented in the outer side of cell membrane, they are able to modify the properties and activities of proteins, occurring side-by-side in the same plasma membrane (*cis*-interaction). Besides that, they can also bind to certain structures expressed on the plasma membranes of bordering cells, thus provide intercellular interactions and signal transductions (*trans*-interaction). Moreover, with the unique features and affinity to complementary molecules, they play an important role as antigen or receptors [1].
Their ability to act as mediator of cell adhesion has been explored within this thesis.

3. *HELICOBACTER PYLORI* THEORETICAL BACKGROUND

3.1. Introduction

3.1.1. History

The fact that the presence of bacteria in stomach may lead to gastric diseases has been known for more than 100 years. The long story of *H. pylori* discovery started in Poland, where Professor of Medicine Walery Jaworski found and described the microorganism with characteristic spiral shape in the dregs of gastric lavage. It was named *Vibrio rugula* and for the very first time designated as a possible cause of gastric disorders. Even though his work was published, it did not spark much interest. Nevertheless, in the first half of the 20th century, similar findings of spiral bacteria in the human stomach, either in post-mortem autopsies or surgical examination, were reported. All these studies were after that challenged by Palmer in 1954, who declared that no spirochetes had been found by him in more than 1100 gastric biopsies. Moreover, the main problem for further investigation of the role of spiral-shape microorganisms in the pathogenesis of diseases affecting human stomach consisted in continual failure to cultivate the collected the bacteria [2]. A real breakthrough came in 1980s, when Warren and Marshall successfully performed the bacterial isolation and culture followed with the self-ingestion experiments demonstrating the colonization and induction of gastric mucosal inflammation as well as the eradication of the infection. Their hard-work was paid off with the awarding of the Nobel Prize in Physiology or Medicine in 2005 "for their discovery of the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease" [3].

3.1.2. Characteristic

The bacterium was originally called a *Campylobacter*-like organism and later changed to *Campylobacter pylori,* as it was categorized into Campylobacter species.

The current name, *Helicobacter pylori*, has been used since it came out to be a member of different genus in 1989 [3].

H. pylori is a gram-negative bacterium, approximately 0.5 – 0.9 μm wide and 2 – 4 μm long. Its appearance can be S-shaped or curved rod, even coccoid-shaped, with 2 – 6 unipolar flagella that provide motility. Each one is sheathed, measures about 3 μm in length and often carry distinctive terminal bulb without any defined function. The coccoid form of *H. pylori* cannot be cultured and it is assumed to represent dead or degenerative cells (Fig. 2).

H. pylori is a microaerophilic organism, thus the conditions for ideal growth include high humidity with 5% level of oxygen and approximately 5 – 10% carbon dioxide. Moreover, the medium optimally contains blood, mostly horse or sheep. The cultures need 3 – 5 days at 37°C to grow the best, though the temperature range is 33 – 40°C depending on the various strains. Despite the fact that the bacterium survives in the acidic environment of stomach, it has been classified as neutralophile with ideal growth at neutral pH. *H. pylori* can produce catalase and oxidase, however its typical and the most important enzyme is urease that may be used for identification of *H. pylori* presence as well [4].

Fig. 2. *Helicobacter pylori*

3.2. Epidemiology and transmission

H. pylori infection occurs worldwide with wide geographical variations. The studies have shown the diversity in prevalence between middle-aged adults in developing countries, which is 80%, and industrialized countries that is slightly lower, 20 – 50%. Moreover, the rate of *H. pylori* infection in industrialized countries has been noticed as significantly decreasing over recent years due to improved sanitation and hygiene habits and antibiotics treatment for other reason as well. The constantly increasing number of *H. pylori* prevalence with age is caused mostly by a cohort effect that reflects higher rates of the infection in the past [5]. It has been confirmed that the socioeconomic conditions,

especially during childhood, play the important role in relation with the contamination risk. Whereas in developing countries the occurrence of *H. pylori* infection rises rapidly in the first 5 years of life and stays high even after, in industrialized countries the prevalence is low in early childhood and gently increases with age.

The exact and detailed mechanisms of *H. pylori* infection have not been properly described and explained. However *H. pylori* is found in some nonhuman primates and other animals, there is no data for zoonotic transfer of infection so far. It has been detected in saliva, vomitus or feces, nevertheless there is still not any clear proof concerning the increased risk of transmission *via* these products. Moreover, *H. pylori* reacts very sensitively to atmospheric oxygen pressure, temperature fluctuation out of the 34 – 40°C range and nutritional stress. Thereby the direct interhuman, mainly intrafamiliar, transmission, *via* both oral-oral and fecal-oral route, is assumed as a most likely source of infection [3].

3.3. Pathogenesis

Despite the fact that the gastric mucosa is relatively well protected against bacterial attacks, *H. pylori* is still able to adapt highly and survive in such hostile environment. It has unique ability to evade the bactericidal activity of the gastric acid and penetrate into the mucus, attach to epithelial cells and start colonizing. Overall, this whole process may result in chronic inflammation leading to atrophy and intestinal metaplasia [6].

Many different factors that affect colonization efficiency in varying degrees, have been described.

3.3.1. Persistence

As *H. pylori* is not an acidophil, an essential requirement for successful long-term colonization in the acidic environment of stomach is the ability to adapt to and even change the ambient environmental conditions. The main mechanism, how *H. pylori* protects itself, is represented by the urease enzyme activity. Urease is able to convert urea into ammonia and carbamate that is subsequently decomposed into carbon dioxide and another molecule of ammonia. As result, the acid-neutralizing cloud of ammonia is formed due to the pH increases and thus bacteria is protected from gastric acidity. Moreover, both products of urease activity participate in the pathogenesis of *H. pylori* infection. It is considered that ammonia has a cytotoxic effect to gastric epithelial cells and beside that, carbon dioxide has negative influence on bactericidal effect of a peroxynitrite. The enzyme activity is controlled by a specific proton-gated urea channel, UreI, opened at low pH until the neutral conditions are set up again [3].

3.3.2. Shape and motility

Once the beneficial conditions of environment have been created, next step is getting through the protective layer of highly viscous gastric mucus. It is thought that *H. pylori* helical shape and flagellum number contribute significantly to bacterial motility enhancing [7]. It has been demonstrated that mutation of *Helicobacter* D,D-peptidasa A (HdpA), which has influence on determining of *H. pylori* shape, causes shape anomaly and thus reduce the potential of bacterial colonization of the gastric mucosa [6]. The urea hydrolysis plays a crucial role one more time, when the induced pH elevation triggers a gastric mucin transition from gel to sol, thereby allows the bacteria to swim easily through the mucus layer [7].

3.3.3. Chemotaxis

The ability of *H. pylori* to swim in the gastric mucus needs to be coupled with the chemotaxis, which ensures the control and correct direction of bacterial movement in response to chemical signals. The presence of transducer-like proteins (Tlps), particles functioning as chemoreceptors in the *H. pylori* membrane or the cytoplasm, provides recognition of chemical ligands from the surroundings. Thereby a signal transduction cascade can be initiated, resulting in direction change of flagellar motors rotation [8]. It was originally thought that *H. pylori* chemotaxis is based on response to the urea and bicarbonate gradients, set up by urease hydrolysis activity in gastric mucus [9]. Afterwards, it was demonstrated that neither urea nor bicarbonate concentrations changes disrupt the spatial orientation of the bacteria. The chemotactic movement toward the gastric epithelium is driven by pH gradient of the environment [10].

3.3.4. Adhesion

Next step, essential for successful colonization and infection, is the *H. pylori* adhesion to the host gastric mucosa, thus it protects from the clearance mechanisms such as liquid flow, peristalsis or shedding and renewal of the mucus layer. The bacterium is able to bind tightly to the epithelium by various surface-bound proteins known as adhesins. These adhesins are expressed on its external and capable to recognize and connect to specific glycan structures exposed on gastric epithelial cells [11].

The best characterized adhesion protein of *H. pylori* is BabA, blood group antigen binding adhesin, which is able to mediate the binding to fucosylated blood group antigens, Lewis b (Le^b), on the host cells surface. Infections caused by *H. pylori* strains with functional BabA have been associated to increased risk of gastric carcinoma incidence.

Another significant molecule termed SabA, sialic acid binding adhesin, enables *H. pylori* binding to mucus layer *via* interaction with sialic acid-containing carbohydrate structures such as sialyl-Lewis x (S-Le^x) and sialyl-Lewis a (S-Le^a). It has been proved that

substitution of nonsialylated Lewis antigens by sialylated correlates with *H. pylori*-induced chronic gastric inflammation and atrophic disease [3].

A number of other outer membrane proteins have relationship to the *H. pylori* adhesion to host epithelial cells, however their participating and importance in the pathogenesis is still under discussions or identifying [11].

3.3.5. Virulence factors

Not every patient, who is infected by *H. pylori*, must contend with the complication that the infection involves. Moreover, they might not experience any obvious clinical symptom at all. This phenomenon is explained as a combination of host-genetic factors, environmental properties and the fact that some strains may be less or more virulent than others. It has been noticed that more virulent strains have the ability to cause changes in morphological features, vacuolization and degeneration of *in vitro*-cultured cells. This improved disease-causing potential is associated with the presence of a protein named CagA, cytotoxin-associated gene A. The *cagA*$^{+}$ strains are associated with the presence of the pathogenicity island (PAI), which was highlighted as the main factor involving an extensive inflammatory response. Cag PAI-encoded proteins coupled with type IV secretion system (T4SS) form a needle-like structure, by which CagA and other bacterial factors can be translocated into the target host cells. After transfer, CagA protein is tyrosine-phosphorylated and thus interacts with various host cell signal molecules. This cascade initiates the morphological changes and proliferation of epithelial cells.

The second major virulence factor having an important role in the *H. pylori* infection pathogenesis is vacuolating cytotoxin (VacA). This exotoxin is able to insert itself to the epithelial cell membrane and to form pores, through that bicarbonate and other anions can be released. It also leads to higher cellular permeability, thus the host cell loose the cations and nutrients. Furthermore, VacA activity is observed in cytosol as well. It accumulates in the inner membrane of mitochondria, stimulates the channels and thereby induces cell apoptosis. This VacA proapoptotic effect targeted to parietal cells may cause in lower secretion of hydrochloric acid, results in development of gastric cancer [3].

Neutrophils and monocytes infiltration into infected gastric mucosa has been noted as a significant step in the process of *H. pylori*-caused inflammation. Based on that observation, 150-kDA *H. pylori* neutrophil-activating protein (HP-NAP) was identified as an important virulence factor, acting as a chemoattractant for inflammatory infiltrates with the ability to induce them to release pro-inflammatory cytokines as well as reactive oxygen radicals and thus contributes to the development of gastritis. Moreover, HP-NAP plays a role of a mediator for *H. pylori* binding to gastric cells since it interacts with carbohydrates structures on cells surface. It occurs in the bacterial cytosol and is released most likely after

cell lysis. Due to HP-NAP immune modulating activity, it has been assumed that this protein may possible become a future tool in vaccination or cancer immunotherapy strategies [12].

The last *H. pylori* virulence factor that is necessary to mention, is outer inflammatory protein A (OipA). In cooperation with other ones, it is responsible for inducing the expression of proinflammatory molecules such as IL-8 [9].

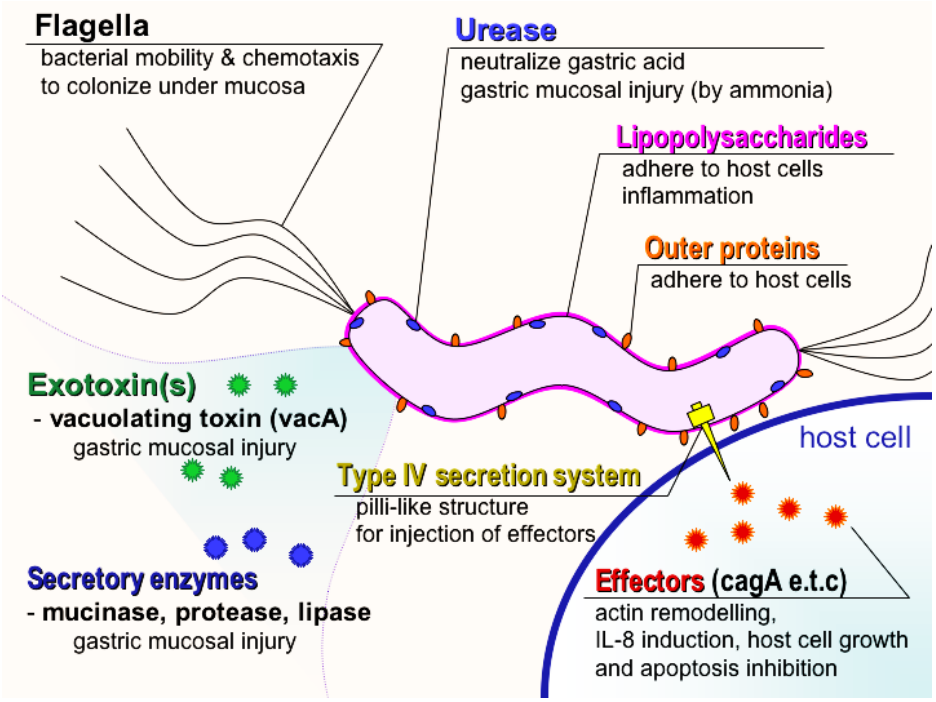

Fig. 3. *Helicobacter pylori* virulence factors

3.4. Diagnosis

Once the patient is infected by *H. pylori*, the key factor for effective treatment is its early diagnosis. Nowadays, there are various currently available methods that can be used for detection of *H. pylori* presence, based on the morphological, immunological, genetics or enzymatic characteristics of the bacteria. All these techniques are usually categorized into non-invasive and invasive tests. Each has its advantages and limitations, therefore the suitable method is chosen based on the symptoms that occurs in the individual patient, age and the patient's ability to undergo the testing, local experiences and clinical setting [3].

3.4.1. Non-invasive testing

This type of testing can be used in patients without alarming symptoms (persistent vomiting, weight loss, gastrointestinal bleeding etc.) and other complications. The tests are carried out with peripheral samples such as blood, stools or breath samples. It includes the urea breath test, serologic assays and stool antigen test [13].

3.4.1.1. Urea breath test

The urea breath test (UBT) represents a highly sensitive and specific method with accuracy about 95% in adults and children over the age of six years. It can be indicated either for the initial diagnosis or for detection of the therapy success. UBT is based on the detection of *H. pylori* urease activity. The execution is very simple and it just needs two breath samples for the evaluation. First one before the testing and the other one approximately 15 – 30 min after drinking the solution containing ^{13}C- or ^{14}C-urea substrate [14]. If the bacteria are present in stomach, orally absorbed ^{13}C- or ^{14}C-labeled urea will be hydrolysed into ammonia and labelled carbon dioxide that is absorbed into the circulation and exhaled by lungs, and is thereby possible to be measured in the exhaled air. The information about the bacterial urease activity is then obtained by comparing the amount of labelled carbon dioxide in the samples [13].

Although the technical equipment for the measuring ^{13}C isotope is quite costly, its non-radioactive and harmless properties, allowing the use of this method in children or pregnant women, still shine. Compared to that, ^{14}C-urea is inexpensive, but involves huge difficulties with radiation exposure, storage and liquidation of hazardous waste [14].

3.4.1.2. Stool antigen test

Stool antigen test (SAT) has become a common non-invasive and quite cheap method that can be well performed in adults and especially in children of all ages. The SAT specificity and sensitivity depend on the setting and whether the assay is performed pre- or post-therapy. In initial diagnoses, it reaches more than 90% and is comparable to the UBT. Nevertheless after treatment, the output of UBT, in some cases, is better than SAT [14]. Overall, SAT has been found to be the most cost-effective method that can cut down the future potential for peptic ulcer disease and gastric cancer [15].

The specimens are usually collected without any problems, however for some patient it could pose an unpleasant situation to take a fecal sample. Furthermore, a decreasing SAT sensitivity appears in patients suffering from diarrhoea as the concentration of antigen is diluted. The presence of another disorders of the digestive tract or bleeding ulcers may affect the SAT results as well. The *Helicobacter*-specific antigen is recognized with the aid of an enzyme immunoassay or immunochromatography [13].

3.4.1.3. Serology

H. pylori serologic testing is based on detecting IgG. It is an inexpensive, widely used method for the diagnosis with the sensitivity and specificity values ranging from 75 – 95%. This method involves the necessity of local validation due to the *H. pylori* strain differences among various geographic locations and prevalence of the infection. Thereby for absolute certainty, positive serology outcomes should be confirmed by UBT or STA.

On the other hand, serologic testing do not deliver false-negative results in patients treated with antibiotics and proton pump inhibitors or struggling with atrophic gastritis or intestinal metaplasia that are characterized by low colonization density [13]. Moreover, concrete virulence factors such as CagA or VacA can be recognized by choosing specific antibodies for testing. The main drawback is the persistence of antibodies in the serum even after effective eradication, which thus limits the usefulness of this technique to check the success of therapy [14]. Despite of different limitations and recommendations, serologic testing is still the choice number one for diagnostic test in the USA [15].

3.4.2. Invasive testing

This kind of testing is indicated in cases, in that the alarming symptoms such as anaemia, gastrointestinal bleeding and weight loss occur, patient is more than 50 years old or not responding to the therapy as well. It combines endoscopy, which allows observing the cellular morphology of gastric mucosa and detecting several structures in real time, with a biopsy followed by urease test on the obtained sample [5].

3.4.2.1. Histology

Histology not only detects the presence of *H. pylori*, it also comes up with further crucial information about the severity level of inflammation and pathological state including atrophy, metaplasia and malignancy. It is costly, time consuming and requires experienced professionals for either biopsy, sample testing or results interpretation [13]. There is a gold standard for gastric biopsy sampling with 5 various specimens from different sites of the stomach, but barely used in everyday practice due to large amount of uncomfortable biopsies. However increasing number of samples can provide better sensitivity of histological testing despite the patchy distribution of bacteria, reduce false negatives and errors during the procedure. *H. pylori* is identified by usual staining with hematoxylin and eosin, or special one, if it is necessary, such as for example Giemsa, Genta or Warthin-Starry silver stain [14].

3.4.2.2. Immunohistology

Immunohistology is more sensitive and specific method for *H. pylori* detection, although not applied to all specimens. It may play an important role in the cases with a serious suspicion for inflammation caused by *H. pylori*, but without any visible presence of bacteria. Besides that, its high specificity guarantees the elimination of other microorganisms with similar morphology that can distort the results [13].

3.4.2.3. Rapid urease test

Rapid urease test (RUT) is based on the specific, well-known urease activity. Its main advantages are simplicity, speed, low costs and specificity. The biopsy sample is placed in a gel containing urea. Once the *H. pylori* is presented, the urease enzyme hydrolyses the urea into ammonia and carbon dioxide results in pH elevation, which is indicated by colour change of the pH indicator. According to the amount of bacteria in the sample, the RUT procedure can take minutes up to 24 h. However the specificity is reduced with increasing incubation time thus occurs the threat of false-positive results.

Nevertheless, even RUT may produce false-positive results, if any other urease-positive bacteria are present in the biopsy. They are usually part of oropharynx microflora swallowed in the saliva, but except the patients with achlorhydria, their urease enzyme with lower activity is disabled by the strong acidity of gastric environment. Furthermore, high probability of false-negative outcomes is related to the recent administration of antibiotics, proton pump inhibitors or bismuth compounds. Decreasing accuracy of the test have been noticed in the presence of blood as well. Generally, the sensitivity of RUT is approximately 85% and more and the specificity higher than 95% [14].

3.4.2.4. Culture

Culture of *H. pylori* from biopsy is not a routine method for early diagnosis, since it is more complicated and time consuming. Anyway, it comes spectacularly into play usually after the malfunction of second-line therapy, because the isolation of *H. pylori* by culture then permits the antibiotic-sensitivity testing. Thus, the most effective treatment can be chosen and administered. Nowadays with standard therapies failure and the antibiotic resistance rates still rising, it may become more often and widely used. The procedures *for H. pylori* antibiotic-sensitivity tests have been worked out by the National Committee for Clinical Laboratory Standards [5]. Moreover, the specimens from the string test or gastric juice sampling can be used as well. These techniques are less aggressive compared to biopsy, though the susceptibility of testing is lower. The bacteriological cultures need to be incubated as soon as possible under appropriate conditions for several days. Even though this represents the most specific method for *H. pylori* detection, the results hinge on the sample quality, using media and microbiologist's skills and experience [14].

3.4.3. Polymerase chain reaction

Real-time polymerase chain reaction (qPCR) can be performed for detection and closer description of *H. pylori* strains*,* moreover assists in identification of bacterial genotypes and specific mutations related to antibiotic resistance such as in clarithromycin- or fluoroquinolone-resistant cases [13]. Due to high sensitivity, it is possible to use non-invasively obtained specimens such as saliva, urine, stool or even a dental plaque.

Its significant drawback includes the segment detection of even dead bacterium that remains in gastric mucosa after treatment, thus delivers false-positive results [14]. However PCR-based testing is still not generally executed because of financial cost, wide applications in both pre- and post-treatment setting with further option of resistant strains recognizing represent a promising diagnostic method in the future [13].

3.5. Clinical outcomes

The clinical manifestation of *H. pylori* infection is affected by many factors associated either with bacteria, host or environmental conditions. Furthermore, no clinical symptoms can be actually observed in many infected individuals. Nevertheless, the bacterium represents a main agent causing chronic gastritis that leads to 10% lifetime risk of ulcer disease outbreak, 1 – 3% likelihood of progress to gastric cancer and 0.1% chance to develop gastric mucosa-associated lymphoid tissue (MALT) lymphoma [6].

3.5.1. Acute and chronic gastritis

H. pylori colonization of the gastric mucosa can develop in mucosal inflammation, which results in infiltration of neutrophils and lymphocytes. The affected areas may be either antrum or corpus, or even both of them termed pangastritis. The most often temporary symptoms in acute phase then include nausea, vomitus or fullness. It can be followed with months long-lasting hypochlorhydria [3].

Without any eradication therapy, initial acute gastritis may develop into chronic form of disease with progression to atrophy. Mucosal atrophy manifests as loss of regular functional glands results in modification of gastric secretion, therefore the levels of stomach acid, pepsinogen or intrinsic factor are disrupted [16]. Moreover, there is a contradictory correlation between the effect of acid on bacterial growing and the bacterial activity results in mucosal inflammation due to excretion of acid is affected. Thereby, final form of *H. pylori* infection is based on that interaction [3] (Fig. 4).

The atrophy changes depend, besides the *H. pylori* persistence, on other elements such as genetic, additional gastrointestinal disorders, low socio-economic status or live style involving inappropriate diet combined with smoking and alcohol. The prevalence of chronic gastritis generally increase significantly with age, however some variances in prevalence within young population occur in different parts of the world [16].

Otherwise, mucosal inflammation can be also caused by autoimmune disorders (such as Crohn’s disease), cytomegalovirus and alcohol or medicaments abuse (such as PPI’s, NSAID’s), but that goes beyond the scope of this thesis [3].

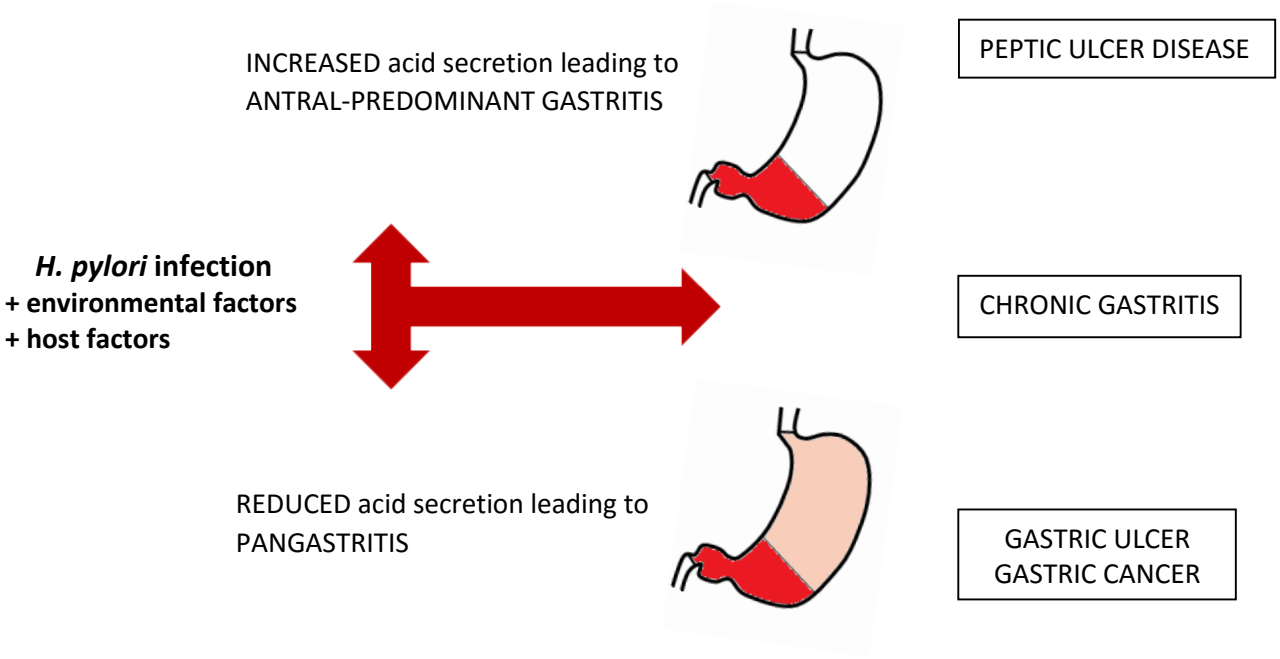

Fig. 4. The correlation between acid secretion and clinical outcome of *H. pylori* infection [3]

3.5.2. Gastric and peptic ulcer disease

Both gastric and peptic (duodenal) ulcers are generally described as a defects in mucosa that extend through the *lamina muscularis mucosae* with a range of at least 5 mm. Gastric ulcers are usually found on the stomach lesser curvature, on the border of corpus and antrum. Peptic ulcers are on the other hand mostly localized in the duodenal bulb [3]. *H. pylori* caused inflammation is responsible for most of duodenal and gastric ulcers, except those with other origin of disease such as for example Zollinger-Ellison syndrome. The pathogenesis is influenced by a number of independent factors, leading by *H. pylori* gastritis and increased acid secretion with smoking, non-secretor status, gender or blood group 0 in the background. Moreover, all these elements have cumulative effect, thereby may result in a very high possibility of ulceration for some individuals [16].

Gastric ulcers are the outcomes of long-term low acid production due to chronic pangastritis initiated by *H. pylori* [3]. Compared to that, peptic ulcers are caused by stomach acid hypersecretion. The inflammation mainly localized in the antrum, the non-acid secreting area, stimulates the excretion of gastrin. The increased amount of gastrin thus incites extra secretion of acid by parietal cells in fundus and corpus. Thereby, elevated acid level damages the already weakened duodenal mucosa results in ulceration and metaplasia [17].

Ulcers disease complications commonly comprise bleeding or perforation, according to the ulcer localization. Bleeding is a typical problem of posterior ulcers, once it grows deep enough to perforate the blood vessel. It occurs in approximately 20% cases.

As the first step, the endoscopic therapy helps to find out the source of bleeding including adrenalin injection, coagulation or the bleeding vessel clipping. Furthermore, the secretion of gastric acid needs to be inhibited, thus PPI's are administered. Secondary, the treatment is closely focused on the cause of bleeding that means the eradication of *H. pylori* infection, which also reduces the likelihood of relapse. On the other hand, ulcers placed on the anterior side may end in perforation, require surgical intervention followed with acid suppression and treatment against *H. pylori* as well. Otherwise, relapse of ulcer disease can result in scarring with a formation of strictures and obstruction. Although the eradication therapy generally cures either strictures or inflammation with oedema, in certain cases the endoscopic balloon dilatation or even surgery need to be performed. Furthermore, suspicion of the stricture formation as outcome of malignancy always must be excluded first [3].

3.5.3. Non-ulcer dyspepsia

Non-ulcer dyspepsia (NUD) is usually defined as discomfort or pain, chronic or recurrent, localized in the centre of upper abdomen without any noticeable structural anomaly. Moreover, it may be accompanied by heartburn or regurgitation, which are, in contrast, the characteristic symptoms of GERD. It has become a frequent complaint, since about 15 – 40% of Western world adult population encounter dyspeptic syndrome. The pathophysiology of NUD hasn't been described sufficiently, however it is presumably related to changes in secretion, motility or susceptibility of gastrointestinal tract with possible participation of *H. pylori* infection as it causes chronic inflammation and thereby affects the gastric acid excretion. Although many different studies have been focused on the NUD-*H. pylori* relation and the efficiency of *H. pylori* eradication therapy to NUD over the years, there is still no united and clear conclusion [18].

Overall, *H. pylori* has a certain influence on the emergence of NUD. Thus, the strategies include non-invasive diagnostic methods followed by acid-suppressive therapy, in the patient without alarming signs or other complications, represent an appropriate and cost-effective option to cure NUD. Moreover, *H. pylori* eradication could help in the prevention of ulcer disease occurrence as well [3].

3.5.4. Gastric mucosa-associated lymphoid tissue (MALT) lymphoma

The lymphoid tissue is not usually present in gastric environment. However, rarely, the population of B cells may rapidly proliferate and slowly form a MALT lymphoma. It is strongly associated with the occurrence of *H. pylori* in stomach, since nearly all of lymphomas patients are infected with *H. pylori*. Moreover, up to 80% of cases respond positively to the eradication of the infection and reach almost complete restoration, about 10% further record some residual signs. The rest of treating patients are resistant to the therapy, which strongly correlates with a certain genetic anomaly in the host [3].

3.5.5. Gastric cancer

H. pylori itself is not a source of carcinogens, but chronic gastric inflammation induced by *H. pylori* may lead to atrophy and intestinal metaplasia. The loss of normally functional cells, reconstruction of mucosal architecture in stomach with replacing by intestinal-like epithelium and fibrotic tissue are the elements considerably increasing the risk of gastric cancer. The renewal of the gastric epithelium is supported and enhanced even more by gastritis, which can stimulate the proliferation of malignant epithelial cells and tumour forming. Some studies indicate that patients with severe and extensive atrophy, may have nearly 90 times higher possibility of gastric cancer outbreak compared to those with no gastric atrophy at all. Furthermore, the risk increases further in individuals suffering from pernicious anaemia in addition [16].

Gastric cancer is the second leading cause of cancer-related death worldwide. Based on the evidence of *H. pylori* infection increasing the risk of gastric cancer, since 1994, *H. pylori* has been classified as a type I carcinogen [5]. The etiology includes the interplay of various factors. Firstly, the bacterial ones affect the gastritis development and severity. It has been demonstrated that the individuals with *cagA*$^+$ strains are more vulnerable. Moreover, the common environmental issue such as diet, alcohol, vitamins and minerals deficiency or low socio-economic status have a certain effect as well. Thus, the prevention and *H. pylori* early eradication therapy play a crucial role in research in this field [3].

3.6. Other related gastrointestinal diseases

Even though the role of *H. pylori* infection is mostly mentioned in connection with the diseases, which were described above, it has been observed in many epidemiologic studies that it may affect even other organs of gastrointestinal tract.

3.6.1. Gastroesophageal reflux disease (GERD)

The eradication of *H. pylori* is key factor in treatment and prevention of ulceration and gastric cancer. However, it can become a problem for patients suffering from gastroesophageal reflux disease. It has been discussed that the presence of *H. pylori* in the stomach may even have a benefit to the host, while it can play a role in protection of GERD development in certain way. The theory is based on the fact that *H. pylori*-induced gastritis leads to decreasing of stomach acid secretion, which may reduce the risk of GERD as well. This hypothesis emerges from a number of observations showing a low prevalence of *H. pylori* among GERD patients with reverse either time or geographical tendency. Nevertheless, it is necessary to confirm that fact by more prospective studies. Otherwise, concerns about potential GERD development do not represent a barrier for a decision of *H. pylori* eradication [3].

3.6.2. Oesophageal malignancies

Nowadays, oesophageal cancer has become the eighth most common cancer in the world. There are two forms differing mainly in histological features, but the diversity in geographic and demographic distribution is evident as well. Oesophageal squamous cell carcinoma (ESCC) comprises the majority of oesophageal cancer cases compared to oesophageal adenocarcinoma (EAC). The association between both ESCC and EAC with the colonization of *H. pylori* has been suggested and studied in various meta-analysis over the years. It was reported that the bacterial presence in stomach, particularly *cagA*$^+$ strains, may have a protective effect against EAC [19]. Similar results were accomplished in another study later. Furthermore, it also included the possible connections between *H. pylori* and ESCC in Asian and non-Asian populations [20].

The reason for the dissimilar influence of *H. pylori* colonization to ESCC or EAC may consist in their different pathogenesis. Barrett's oesophagus is known as crucial element in EAC development, as it leads to repeated gastroesophageal reflux and chronic inflammation followed by ulceration and ends in epithelial reconstruction. It has been reported that the radical eradication of *H. pylori* infection may be a risk factor for GERD and thus for EAC. Besides the amount of gastric acid, it may bring the elevated level of ghrelin in serum as well, thus resulting in increased rate of obesity and impaired function of oesophageal sphincter.

Furthermore, in closer location analysis, it was found that *cagA*$^+$ strains have inverse relation to ESCC in Asian population, but positive connection to non-Asian ones. ESCC risk factors comprise smoking, alcohol consumption, unhealthy diet with high salt and low vegetables intake and long-term mucosal irritation. Therefore, the diverse association of ESCC to Asian and non-Asian populations may involve in different diet habit and lifestyle. Moreover, either inverse or positive relation between *cagA*$^+$ *H. pylori* strains and ESCC could be explained somehow. The influence of lower level of stomach acid due to gastric atrophy and *H. pylori*-induced apoptosis of ESCC cells on the one side, *N*-nitroso compounds produced by bacterial colonies as a factor for oesophageal cancer development on the other. All these hypothesis need to be confirmed or disproved by further studies [20].

3.6.3. Pancreatic cancer

Pancreatic cancer has become one of the leading causes of cancer mortality worldwide and its etiology and risk factors have not been really well understood. Despite a series of various studies, a question whether *H. pylori* infection may negatively affect the risk of pancreatic cancer development has not been clearly answered yet. Some of them assume that the presence of *H. pylori* in stomach, particularly the more virulent *cagA*$^+$ strains, may be reflected on higher risk of pancreatic cancer similarly as it is known to have impact on gastric cancer progression. The mechanism could consist in increased excretion of gastrin and lower level of somatostatin due to *H. pylori*-induced gastritis. This should lead to

enlargement of the pancreas and higher sensitivity to carcinogens. Moreover, pernicious anaemia, which has close relation to hypergastrinemia, has been connected to pancreatic cancer. Besides that, increased amount of *N*-nitrosamines as an output of bacterial overgrowth in stomach could be a next risk factor [21]. Against it, some researcher reported that no significant association between *H. pylori* colonization and increased risk of pancreatic cancer development has been found [22]. Anyway, additional studies needed to be performed to clarify this issue.

3.6.4. Colorectal neoplasms

Similarly to the cases mentioned above, an association between *H. pylori* and the possibility of elevating the colorectal cancer risk has been the focus of several studies. It has been demonstrated that *H. pylori* infections with *cagA*+ strains are connected to increased risk for colorectal carcinogenesis compared to infection with *cagA*- strains. The finding that *cagA*$^{+}$ strains cause higher production of gastrin than *cagA*$^{-}$ strains, thereby leading to hypergastrinemia, which is known as a risk factor, supported this hypothesis. Furthermore, it was suggested that the bacteria may contact the colonic mucosa, where the CagA protein could induce cytokines expression (*e.g.* interleukin-8) directly in the colon and thereby triggers the cancerogenesis [23]. Recently, the similar conclusion with the negative effect of *H. pylori cag*$^{+}$ strains has been reported. However, the connection between hypergastrinemia and higher risk for colonic neoplasia was not found in this study [24]. Related to another analysis presenting a lack of *H. pylori*-colorectal cancer association in certain populations, further studies need to be done to confirm the *H. pylori* influence or eventual population dependence [25].

3.6.5. Hepatobiliary issues

A hypothesis about *H. pylori* possible impact to hepatobiliary disorders stems from the finding of *H. pylori* and other *Helicobacter* species in gallstone, bile juice and hepatobiliary tissues [26]. It has been assumed that the bacterium could reach the liver *via* blood or biliary system. However, it is unclear whether *H. pylori* may play a role in pathogenesis of liver diseases or whether its presence in hepatobiliary system is just a secondary outcome of existing diseases. Furthermore, many investigations have proved the bacterial influence in the development of disorders, such as non-alcoholic fatty liver disease, liver fibrosis, liver inflammation etc., but some of them have shown exact the opposite. The eventual mechanisms have not been found and described. Thus, the role of *H. pylori* in pathogenesis of hepatobiliary diseases still remains speculative [27].

3.7. Related extragastric diseases

H. pylori was primarily attributed to development of gastrointestinal diseases, although recently it has been shown that its influence ranges even over those borders, including cardiovascular, neurological or respiratory tracts.

3.7.1. Cardiovascular diseases

Several studies have pointed out the association between *H. pylori* infection, particularly *cagA*$^+$ strains, and significantly higher risk of cardiovascular diseases. Whereas an inflammation may negatively affect the destabilization of atherosclerotic plaques, increased levels of interleukin-6 in serum may have an impact to ischemic heart disease [28]. The studies focused on an eventual connection to stroke have delivered different conclusions. However, it could consist of diverse mechanisms of embolic and ischemic stroke pathogenesis [29].

3.7.2. Diabetes mellitus

The possible role of *H. pylori* in diabetes mellitus (DM) has been studied as well. A number of studies have reported a relation of chronic *H. pylori* infections with elevated levels of glycated haemoglobin, reduced levels of insulin and insulin resistance. Moreover, positive correlations to other DM complications, such as nephropathy or neuropathy, have been observed. On the other hand, some analyses have come to opposite results [28].

3.7.3. Haematological issues

H. pylori infection impact to iron deficiency anaemia (IDA) has been explored. Strong evidences of *H. pylori*-IDA correlation have been reported, such as complete recovery from IDA due to eradication therapy or lower responses to iron supplementation in IDA‘s patients with active infection compared to patients without it. The mechanism may include an elevation of hepcidin serum levels, which is a regulator inhibiting iron entry to plasma, thus reducing the response to iron therapy. Nevertheless, further research has to be performed to confirm that fact as some studies did not support the connection [28].

A linkage between *H. pylori* and idiopathic thrombocytopenic purpura (ITP) has been recently confirmed. Significant positive effect of bacterial eradication to ITP patient’s condition has been also reported. However, as it was concluded that *H. pylori* infection itself cannot cause IDA or ITP, additional factors may play a role as well [29].

3.7.4. Neurological disorders

Besides an unclear influence of *H. pylori* infection to stroke, an association to dementia has been also investigated. In the report, it has been explained that the infection could induce responses of either humoral or cellular immunity leading to interactions with

host's nervous tissue and thus could cause nerve cell destructions. Moreover, some experiments have shown the positive impact of *H. pylori* elimination to Alzheimer disease manifestations. Apart from Alzheimer disease, the relations to Parkinson's disease, multiple sclerosis or posthepatic encephalopathy have been studied recently [29].

3.7.5. Others

A series of other disorders have been associated to *H. pylori* infection, such as dermatological diseases, open-angle glaucoma, inflammatory bowel disease and pregnancy-related diseases. *H. pylori* has also been identified in some specimens from respiratory tract. However, more additional studies are needed to understand better the possible mechanisms of *H. pylori* infection influence [28, 29].

3.8. Treatment and vaccination

As it has been described above, *H. pylori* infection can cause a number of unpleasant health troubles. Therefore, the main aim of therapy is bacterial eradication and thus reduction of likelihood of reinfection to a minimum. A series of *H. pylori*-treatment schemes have been assembled. However, the clinically significant are those, which deliver the cure rates at least 80% with no occurrence of serious side effects and minimal initiation of bacterial resistance. Furthermore, the acidity of gastric environment represents an issue, as it may affect the effectiveness of antibiotics and thereby spoil the cure. Therefore it is necessary to combine antimicrobial chemotherapy with either proton pump inhibitors or bismuth compounds. Various combinations of two antimicrobial and one antisecretory agent, called triple therapies, administered for 7 – 14 days have been studied and approved by FDA (Table 2). Administration of two or more antibiotics supports the therapy effect and decreases the possibility of *H. pylori* resistance. Clarithromycin, amoxicillin, metronidazole and tetracycline are the most widely prescribed antibiotics. A frequency of clarithromycin and metronidazole resistance is substantially higher compared to the others, therefore substitutions by tinidazole, rifabutin or even ciprofloxacin have been also reported.

First-line treatment typically involves triple therapy, consisting of amoxicillin, clarithromycin and PPI (such as lansoprazole, omeprazole, esomeprazole or pantoprazole) administered for 7 – 14 days. Metronidazole is otherwise an alternative option in patients allergic to penicillins. Furthermore, the substitution of PPI by bismuth subsalicylate or just dual therapy including clarithromycin with ranitidine bismuth citrate is approved and promoted as well [5].

FDA-APPROVED TREATMENT OPTIONS FOR *H. PYLORI* ERADICATION
Omeprazole (40 mg QD) + Clarithromycin (500 mg TID) for 2 weeks, then Omeprazole (20 mg QD) for 2 weeks
Omeprazole (20 mg BID) + Clarithromycin (500 mg BID) + Amoxicillin (1 g BID) for 10 days
Lansoprazole (30 mg BID) + Clarithromycin (500 mg BID) + Amoxicillin (1 g BID) for 10 days
Lansoprazole (30 mg BID) + Amoxicillin (1 g BID) + Clarithromycin (500 mg TID) for 10 days
Lansoprazole (30 mg TID) + Amoxicillin (1 g TID) for 2 weeks [1]
Esomeprazole (40 mg QD) + Clarithromycin (500 mg BID) + Amoxicillin (1 g BID) for 10 days
Ranitidine bismuth citrate (400 mg BID) + Clarithromycin (500 mg TID) for 2 weeks, then Ranitidine bismuth citrate (400 mg BID) for 2 weeks
Ranitidine bismuth citrate (400 mg BID) + Clarithromycin (500 mg BID) for 2 weeks, then Ranitidine bismuth citrate (400 mg BID) for 2 weeks
Bismuth subsalicylate (525 mg QID) + Metronidazole (250 mg QID) + Tetracycline (500 mg QID) [2] for 2 weeks + H_2-receptor antagonist therapy as directed for 4 weeks

[1] This dual-therapy regimen has restrictive labelling. It is indicated for patients who are either allergic or intolerant to clarithromycin or for infections with known or suspected resistance to clarithromycin.
[2] Although not approved by the FDA for this indication, amoxicillin has been substituted for tetracycline in patients for whom tetracycline is not recommended.

QD = every day, BID = twice daily, TID = three times a day, QID = four times a day

Table 2. FDA-approved treatment options for *H. pylori* eradication [5]

If an initial cure-strategy fails, second-line therapy will have to be established. That situation usually occurs due to antibiotic resistance, most commonly to clarithromycin, or patient's non-compliance. Quadruple therapy combines PPI, bismuth subsalicylate with metronidazole and tetracycline for 10 – 14 days. Other treatment regimens based on multistep drug-combined administration as sequential and hybrid therapy, or concomitant therapy comprising simultaneous use of three antibiotics with PPI, can be chosen in correlation to different rate of clarithromycin resistance in different areas, to availability of certain drugs and to patient's compliance. In case that both, first-line as well as second-line, strategy fail, eventual third-line treatment then includes other antimicrobial chemotherapeutics as rifabutin, ciprofloxacin or furazolidone.

H. pylori elimination during pregnancy or lactation should be postponed due to contraindication of some medicaments. The therapy should be based only on acid suppression.

It has been also observed, that the cure can be supported by probiotics containing *Lactobacillus* and *Bifidobacterium*, as they may directly disturb bacterial adherence or produce antimicrobial agents. It does not only enhance the eradication rate, but also activates host' immune system and reduces total side effects [14].

Besides research focused on effective *H. pylori* eradication and therapy, vaccines development has been investigated in the last decade as well. It would represent a valuable tool in gastric cancer protection and decrease the risk of antibiotic resistance in *H. pylori* due to less frequent bacterial exposure to antimicrobial agents. However, the development has encountered numerous obstacles with immune response and vaccine efficacy along with pharmaceutical companies' interest and investment. Nevertheless, not only vaccination has an impact in prevention against *H. pylori*. Low socioeconomic status as well as living conditions and hygiene habits are commonly known as risk factors. Therefore, upgrade of these aspects could also play a great preventive role. In summary, further investigations need to be performed to achieve desired results in this field [3].

4. AIM

As the bacterial adhesion to host's gastric mucosa represents a crucial step in successful colonization of *H. pylori*, this thesis was focused on the recognition and structural characterization of SabA binding gangliosides isolated from the total acid fraction of human liver metastasis from lung cancer.

5. MATERIALS AND METHODS

5.1. Purification of gangliosides

5.1.1. Silica gel column chromatography

Firstly, approximately 1.9 g of the total acid glycosphingolipids fraction of human liver metastasis from lung cancer was purified on column chromatography using Silica gel S (Riedel-de Haën, Seelze, Germany). The fractions were eluted by increasing ratio of methanol to chloroform, beginning with 5% of methanol in chloroform, and ending with 100% methanol. 557 mg of GSL mixture was obtained and used for further separation.

For the next separations, Iatrobeads silica gel (Iatrobeads 6RS-8060, Iatron Laboratories, Tokyo) was used as the stationary phase, since it has finer particles than the previous type of silica gel, better purification was achieved. For this separation, the mixture chloroform-methanol-water (60:35:8, v/v/v) was chosen as a mobile phase. Chromatography process was continuously checked by TLC followed by visualization with anisaldehyde or resorcinol staining. In the end, the fractions were pooled according to their mobility on TLC. Each fraction was characterized by LC-ESI/MS. Based on these results, fractions for *H. pylori* binding assays and for further separation were selected.

5.1.2. Thin layer chromatography

5.1.2.1. Application of samples and development of chromatograms

4 – 20 μg of GSLs mixtures as the samples were applied approximately 15 mm from the bottom of the glass or aluminium backed silica gel 60 HPTLC plate (Merck, Darmstadt, Germany) by adequate microsyringe as series of droplets to get 5 – 10 mm lane. The frequently used developing solvents were mixtures of chloroform-methanol-water (60:35:8, v/v/v) or chloroform-methanol-0.25% KCl (50:40:10, v/v/v).

5.1.2.2. Staining and quantification

A number of different reagents can be used for gangliosides detection.

Universal anisaldehyde-sulfuric acid spraying is applicable for recognition of various natural compound as carbohydrates, phenols, glycosides etc. It allows detection of both glycosphingolipids and other compounds in a sample. Glycosphingolipids are coloured green by anisaldehyde staining, while contaminants, as *e.g.* phospholipids, are coloured blue or pink. The solution was prepared by mixing of 1 ml 4-methoxybenzaldehyde (anisaldehyde) with 98 ml glacial acetic acid and 2 ml concentrated sulfuric acid [30].

Resorcinol reagent was used for distinction of gangliosides containing sialic acid. Sialylated gangliosides produced a characteristic blue-violet colour, whereas neutral or sulphated GSLs were visualized by less intense yellow-brown colour [31]. The preparation of the reagent started with dissolving of 2 g resorcinol in 100 ml water. Then 10 ml of this solution was mixed with 80 ml concentrated hydrochloric acid and 0.25 ml of 0.1 M copper sulphate [32].

To make sure that the plate is completely dry before the staining procedure, the rest of solvent was removed by slight heating with a hair dryer. Then the plate was positioned upright in a spray box. The reagent was applied with a fine mist sprayer, moving in a zigzag pattern until the plate was evenly sufficiently moistened. In case that resorcinol mixture was used, the sprayed chromatogram needed to be covered with a clean, equal size glass plate. After that, it was placed in the oven as long as the strains were recognized. Overheating could cause the discoloration of the gangliosides [31].

5.2. Chromatogram binding assay

5.2.1. General procedure

The aluminium backed HPTLC plates needed to be preeluted with methanol before the sample applying. After the chromatography, there was a necessity to make a plastic fixation by putting the dried chromatograms into the mixture of 0.5% polyisobutylmethacrylate (Aldrich Chemical Company, USA) (w/v) in diethylether/*n*-hexane (1:5, v/v) for 1 minute and then let them get air-dried [33]. The plastic coating prevents flaking of silica gel from the support during the next steps of the procedure. It is assumed that the hydrophobicity of the protection layer induces a similar presentation of GSLs as in the plasma membrane [31]. Thereafter, the chromatograms were incubated in phosphate buffered saline containing bovine serum albumin (PBS/BSA) for 1.5 h at room temperature. This step reduced nonspecific binding of the ligands. Then the plates were incubated with chosen ligands diluted in PBS/BSA (often 1:100, v/v) for 2 h at room temperature. Finally, the plates were washed 5 times with PBS and allowed to dry. If radiolabelled ligands or secondary antibodies were used, binding was detected after over-night exposure of the plate to Biomax MR film (Carestream Health, New York, USA) [33].

5.2.2. Antibodies

The monoclonal antibodies directed against the NeuAcα3-lacto epitope (clone TR4/SL-50) and the NeuAcα3-neolacto epitope (clone LM1:1a) (Refs 7+8) [34, 35] were kind gifts from Dr. Maria Blomqvist, Department of Clinical Chemistry and Transfusion Medicine, Institute of Biomedicine, Göteborg, Sweden.

After incubating the plate in PBS/BSA for 1.5 h at room temperature as described above (chapter 5.2.1.), 25 µl of antibodies were diluted in 5 ml of PBS/BSA and the plate was covered with that solution for 2 h. The plate, washed 5 times with PBS, subsequently underwent incubation with 100 µl anti-mouse antibodies that was labelled with ^{125}I and diluted in 5 ml of PBS/BSA for 2 h. Next steps were described above (chapter 5.2.1.) (Fig. 5.).

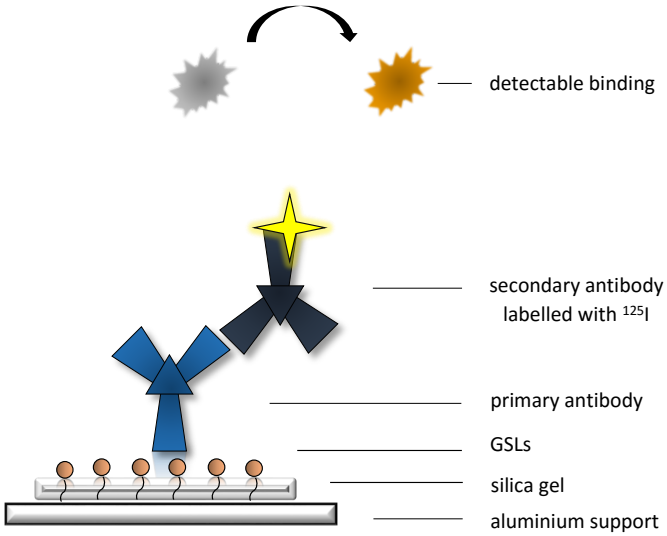

Fig. 5. Chromatogram binding assay with antibodies

5.2.3. Bacteria

Bacterial strains

The SabA-expressing *H. pylori* strain 44777 was from Culture Collection University of Gothenburg. The SabA- and BabA- expressing strain J99, and the J99/SabA knock-out strain, were kind gifts of Dr. Thomas Borén, Umeå. The construction of the SabA deletion mutant has been described (Ref 8) [36].

H. pylori were grown under microaerophilic conditions on Brucella medium (Difco Laboratories, Irvine, California), which contained 10% fetal calf serum (Harlan Sera-Lab, Loughborough, United Kingdom) inactivated in a water bath at 56°C for 1 h, and BBL IsoVitale X Enrichment (Becton Dickson Microbiology Systems, Franklin Lakes, New Jersey).

Next step, comprising the radiolabelling of bacteria, was performed by the addition of 50 μCi ^{35}S-methionine (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) diluted in 0.5 ml PBS, pH 7.3, to the culture plates. The incubation took 12 – 72 h at 37°C and after that the bacteria could be harvest. Moreover, it had to be washed four times by centrifugation at 3500 rpm for 10 min in PBS, pH 7.3. In the end, the bacteria were resuspended in PBS containing 2% (w/v) bovine serum albumin (PBS/BSA) to approximately 1 × 10^8 CFU/ml. The final suspensions had the specific activities approximately 1 cpm per 100 *H. pylori.*

The mutant strain J99/SabA- was cultivated on the same medium with the addition of chloramphenicol (20 μl/ml) [33]. Following procedures were the same as describe above.

Chromatogram binding assays were performed thereafter as it was described above (chapter 5.2.1.) (Fig. 6.).

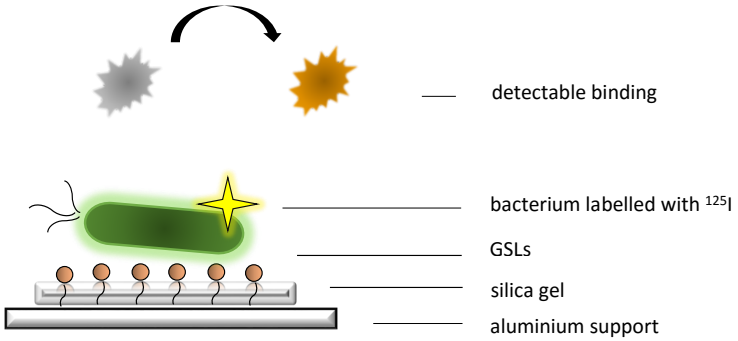

Fig. 6. Chromatogram binding assay with bacteria

5.2.4. Lectins

Aleuria aurantia lectin was purchased from Vector Laboratories, Inc., Burlingame, CA. Aliquots of 100 μg of AAL was labelled with ^{125}I by the Iodogen method according to the manufacturer's instructions (Pierce, Rockford, IL), giving approximately 5000 cpm/μg protein.

After incubating the plate in PBS/BSA for 1.5 h at room temperature as described above (chapter 5.2.1.), 50 μl of lectin, labelled with ^{125}I, were diluted in 5 ml of PBS/BSA and the plate was covered with that solution for 2 h. Following steps were described above (chapter 5.2.1.) (Fig. 7.).

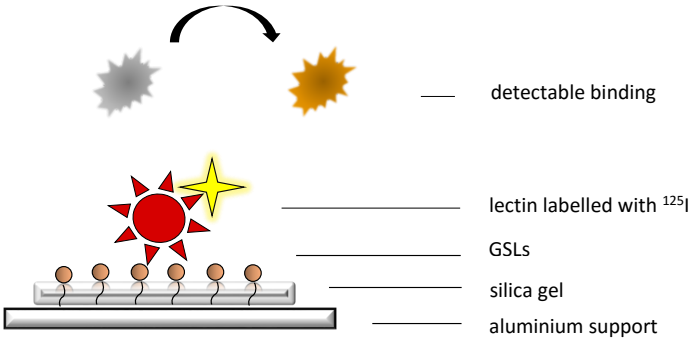

Fig. 7. Chromatogram binding assay with lectins

5.3. Structural analysis of isolated gangliosides

The glycosphingolipids (dissolved in methanol/acetonitrile 75:25, by volume) were separated on a 200 x 0.150 mm column, packed in-house with 5 μm polyamine II particles (YMC Europe GMBH, Dinslaken, Germany), and eluted with a water gradient (A: 100% acetonitrile; B: 10 mM ammonium bicarbonate). Samples were analyzed on an LTQ linear quadrupole ion trap mass spectrometer (Thermo Electron) by LC-ESI/MS at -3.5 kV. Full-scan (*m/z* 500-1800, 2 microscans, maximum 100 ms, target value of 30 000) was performed, followed by data dependent MS^2 scans (2 microscans, maximum 100 ms, target value of 10 000) with normalized collision energy of 35%, an isolation window of 2.5 units, an activation q = 0.25, and an activation time of 30 ms.

Manual assignment of glycosphingolipid sequences was done with the assistance of the Glycoworkbench tool (Version 2.1), and by comparison of retention times and MS^2 spectra of reference glycosphingolipids.

6. RESULTS

Isolation of gangliosides from human lung cancer metastasis

Finally, 178.5 mg of ganglioside-containing fractions were obtained in summary. Each fraction was characterized by LC-ESI/MS, in order to select fractions to be used for *H. pylori* binding experiments.

LC-ESI/MS

The obtained acid glycosphingolipid fractions were analyzed by liquid chromatography electrospray ionization mass spectrometry (LC-ESI/MS). The identity of the glycosphingolipids was obtained by MS/MS (MS^2), based on deduction of the carbohydrate sequence and ceramide composition. By MS^2 of singly charged molecular ($[M-H^+]^-$ ions and doubly charged molecular $[M-2H^+]^{2-}$ ions, the glycosidic bonds between the carbohydrate units were broken, giving rise to Y type ions and C and B type ions (Fig. 8). By calculations using the molecular masses for different types of carbohydrates (fucose 146, hexose 162, *N*-acetylhexosamine 203, and *N*-acetyl neuraminic acid 290), and for different types of ceramides, the carbohydrate sequences of the glycosphingolipids were deduced.

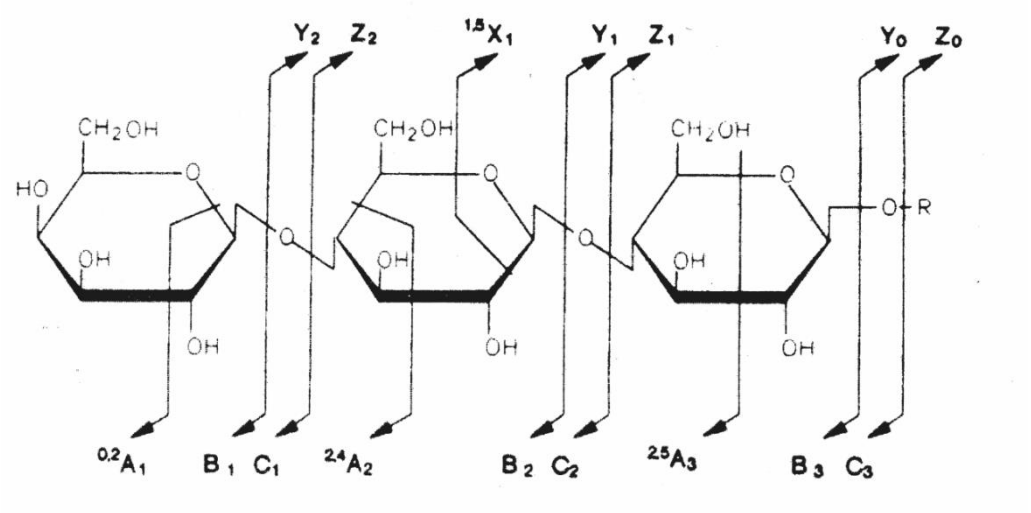

Fig. 8. Product ions arising from fragmentation within the carbohydrate part of glycosphingolipids.

Mass spectrometry analyses of some selected fractions are shown below.

1. Fraction I

In the base peak chromatogram obtained by LC-ESI/MS of fraction I there were two $[M-2H^+]^{2-}$ ions at *m/z* 758, eluted at 20.8 min and 22.3 min, respectively (data not shown). Such $[M-2H^+]^{2-}$ ions corresponds to $[M-H^+]^-$ ions at *m/z* 1517, and indicate gangliosides with one NeuAc, one HexNAc, three Hex, and d18:1-16:0 ceramide. A series of Y ions (Y_0 at *m/z* 536, Y_1 at *m/z* 698, Y_2 at *m/z* 860, Y_3 at *m/z* 1063, Y_4 at *m/z* 1225) was in both cases obtained by MS^2 (Fig. 9). This demonstrated gangliosides with NeuAc-Hex-HexNAc-Hex-Hex sequence and d18:1-16:0 ceramide. In the MS^2 spectrum of *m/z* 758, eluted at 20.8 min there was also a $^{0,2}X_4$ ion at *m/z* 1295 (Fig. 9A). This type of ion is diagnostic of a terminal α6-linked NeuAc [37]. Thus, fraction I was a mixture of gangliosides with NeuAc-Hex-HexNAc-Hex-Hex sequence and terminal α3-linked and α6-linked sialic acids.

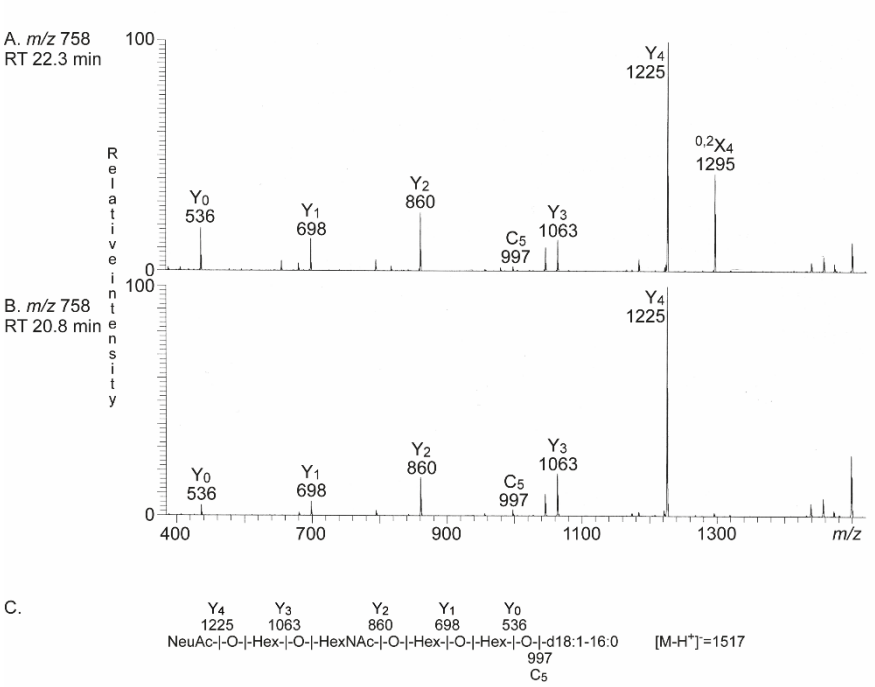

Fig. 9. LC-ESI/MS of the acid glycosphingolipid fraction I isolated from a human lung cancer metastasis.

A. MS^2 spectrum of the $[M-2H^+]^{2-}$ ion at *m/z* 758 (retention time 22.3 min).

B. MS^2 spectrum of the $[M-2H^+]^{2-}$ ion at *m/z* 758 (retention time 20.8 min).

C. Interpretation formula showing the deduced glycosphingolipid sequence.

2. Fraction II

The base peak chromatogram from LC-ESI/MS of fraction II also had a $[M-2H^+]^{2-}$ ion at *m/z* 758, eluted at 20.5 min (data not shown). There was also a $[M-2H^+]^{2-}$ ion at *m/z* 814 (corresponding to a $[M-H^+]^-$ ion at *m/z* 1628).

MS^2 of the $[M-2H^+]^{2-}$ ion at *m/z* 758 gave the same series of series of Y, B- and C-type ions as above, again demonstrating a ganglioside with NeuAc-Hex-HexNAc-Hex-Hex sequence and d18:1-16:0 ceramide. MS^2 of the $[M-2H^+]^{2-}$ ion at *m/z* 814 resulted in Y ion series (Y_0 at *m/z* 648, Y_1 at *m/z* 810, Y_2 at *m/z* 972, Y_3 at *m/z* 1175, and Y_4 at *m/z* 1335) also showing a ganglioside with NeuAc-Hex-HexNAc-Hex-Hex sequence and in this case d18:1-24:0 ceramide. There were no a $^{0,2}X_4$ ions at *m/z* 1295 or at *m/z* 1407, so in both cases the terminal sialic acid was α3-linked.

3. Fraction III

The base peak chromatogram obtained by LC-ESI/MS of fraction III had two $[M-2H^+]^{2-}$ ions at *m/z* 941 and *m/z* 904, respectively (Fig. 10A). The $[M-2H^+]^{2-}$ ion at *m/z* 941 corresponded to a $[M-H^+]^-$ ion at *m/z* 1882, and indicated a ganglioside with one NeuAc, two HexNAc, four Hex, and d18:1-16:0 ceramide. MS^2 of this $[M-2H^+]^{2-}$ ion (Fig. 10B) gave a series of Y ions (Y_0 at *m/z* 536, Y_1 at *m/z* 698, Y_2 at *m/z* 860, Y_3 at *m/z* 1063, Y_4 at *m/z* 1225, Y_5 at *m/z* 1428, and Y_6 at *m/z* 1590), which along with the B- and C-type ions, demonstrated a ganglioside with NeuAc-Hex-HexNAc-Hex-HexNAc-Hex-Hex sequence and d18:1-16:0 ceramide.

The $[M-2H^+]^{2-}$ ion at *m/z* 904 corresponded to a $[M-H^+]^-$ ion at *m/z* 1808, indicating a ganglioside with two NeuAc, one HexNAc, three Hex, and d18:1-16:0 ceramide, and the mass spectrum obtained by MS^2 spectrum of the ion at *m/z* 904 (Fig. 10C) was characteristic for the GD1b ganglioside [38].

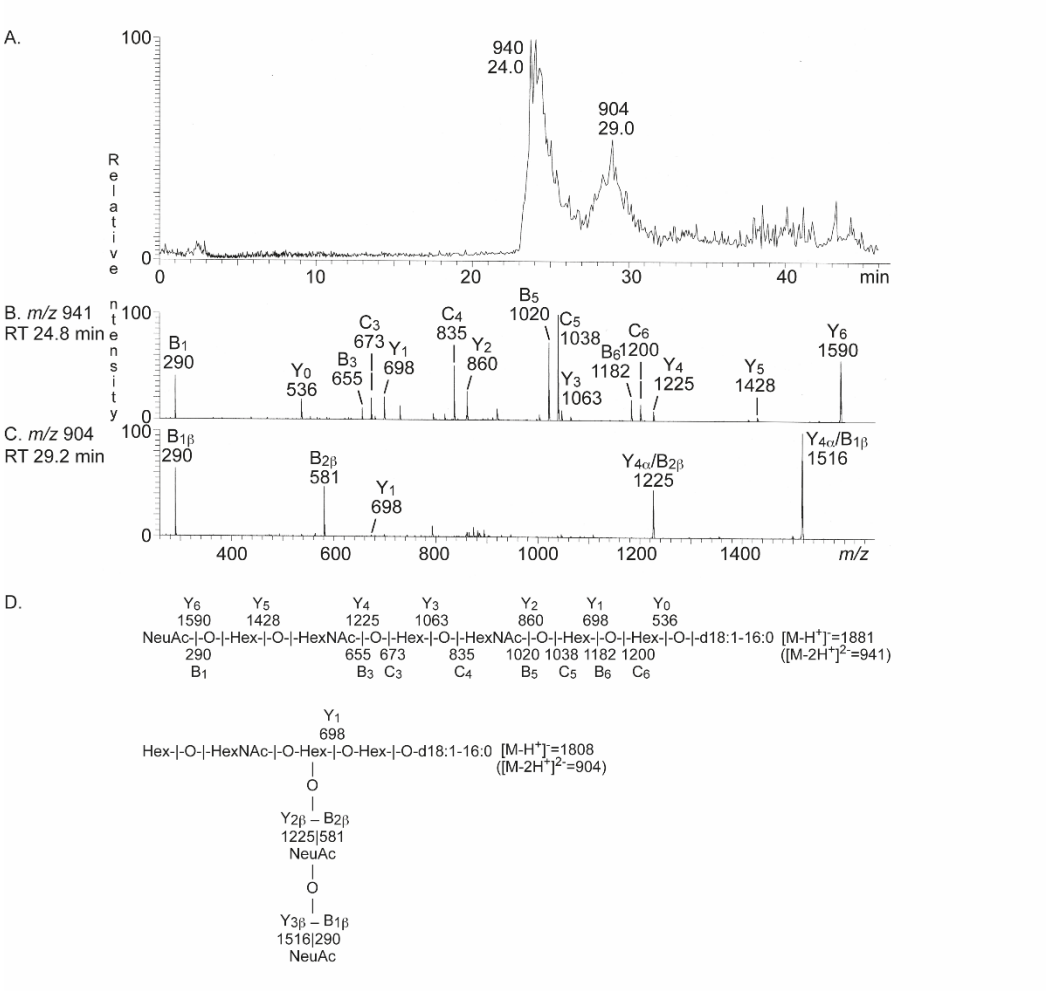

Fig. 10. LC-ESI/MS of the acid glycosphingolipid fraction III isolated from a human lung cancer metastasis.

A. Base peak chromatogram.

B. MS^2 spectrum of the $[M\text{-}2H^+]^{2-}$ ion at *m/z* 941 (retention time 24.8 min).

C. MS^2 spectrum of the $[M\text{-}2H^+]^{2-}$ ion at *m/z* 904 (retention time 29.2 min).

D. Interpretation formulas showing the deduced glycosphingolipid sequences.

4. Fraction IV

Three $[M-2H^+]^{2-}$ ions at *m/z* 948, *m/z* 1022 and *m/z* 911 were present in the base peak chromatogram obtained by LC-ESI/MS of fraction IV (Fig. 11A). The $[M-2H^+]^{2-}$ ion at *m/z* 948 corresponded to a $[M-H^+]^-$ ion at *m/z* 1897, and indicated a ganglioside with one NeuAc, two HexNAc, four Hex, and d18:1-h16:0 ceramide. MS^2 of this $[M-2H^+]^{2-}$ ion (Fig. 11B) gave a series of Y ions (Y_0 at *m/z* 552, Y_1 at *m/z* 714, Y_2 at *m/z* 876, Y_3 at *m/z* 1079, Y_4 at *m/z* 1241, and Y_6 at *m/z* 1506), which along with the B- and C-type ions, demonstrated a ganglioside with NeuAc-Hex-HexNAc-Hex-HexNAc-Hex-Hex sequence, as above, and in this case with d18:1-h16:0 ceramide.

The $[M-2H^+]^{2-}$ ion at *m/z* 1022 corresponded to a $[M-H^+]^-$ ion at *m/z* 2044. Since the mass difference between the ganglioside characterized above corresponds to the mass of a fucose residue this indicated a ganglioside with one NeuAc, one Fuc, two HexNAc, four Hex, and d18:1-16:0 ceramide. The mass spectrum obtained by MS^2 of the ion at *m/z* 1022 (Fig. 11C) had mainly C and B type ions ($B_{1\alpha}$ at *m/z* 290, $C_{2\alpha}$ at *m/z* 470, $C_{3\alpha}$ at *m/z* 673, $C_{4\alpha}$ at *m/z* 835, B_5 at *m/z* 1166, C_5 at *m/z* 1184, C_6 at *m/z* 1346, and C_7 at *m/z* 1508), which in combination with Y ions series (Y_0 at *m/z* 552, Y_1 at *m/z* 714, Y_2 at *m/z* 876, $Y_{5\alpha}$ at *m/z* 590, and $Y_{6\alpha}$ at *m/z* 1752) allowed identification of a ganglioside with NeuAc-Hex-HexNAc-Hex-(Fuc-)HexNAc-Hex-Hex sequence, and with d18:1-h16:0 ceramide.

The $[M-2H^+]^{2-}$ ion at *m/z* 912 corresponded to a $[M-H^+]^-$ ion at *m/z* 1824, indicating a ganglioside with two NeuAc, one HexNAc, three Hex, and d18:1-h16:0 ceramide. As above, the mass spectrum obtained by MS^2 spectrum of the ion at *m/z* 912 (Fig. 11D) was characteristic for the GD1b ganglioside [48].

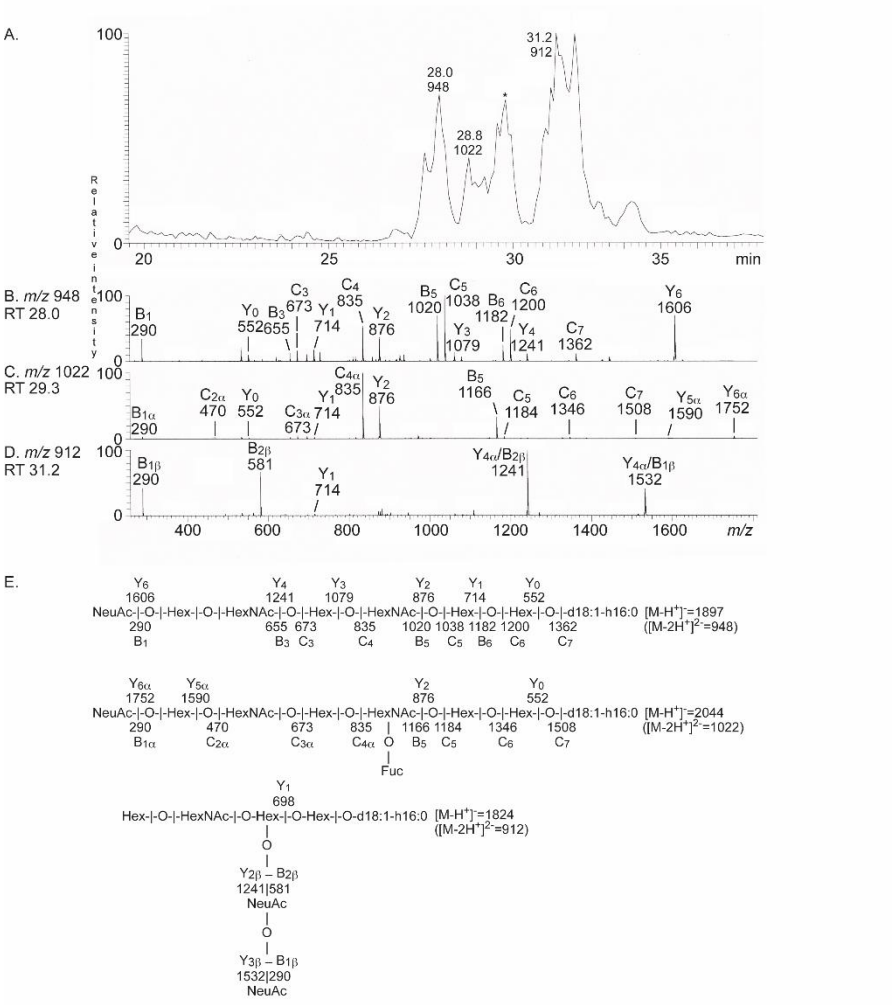

Fig. 11. LC-ESI/MS of the acid glycosphingolipid fraction IV isolated from a human lung cancer metastasis.

A. Base peak chromatogram. The * marks a non-glycosphingolipid contaminant.

B. MS^2 spectrum of the $[M\text{-}2H^+]^{2-}$ ion at *m/z* 948 (retention time 28.0 min).

C. MS^2 spectrum of the $[M\text{-}2H^+]^{2-}$ ion at *m/z* 1022 (retention time 29.3 min).

D. MS^2 spectrum of the $[M\text{-}2H^+]^{2-}$ ion at *m/z* 912 (retention time 31.2 min).

E. Interpretation formulas showing the deduced glycosphingolipid sequences.

5. Fraction V

The base peak chromatogram from LC-ESI/MS of fraction V had three $[M\text{-}2H^+]^{2-}$ ions at *m/z* 948, *m/z* 1123 and *m/z* 912, respectively (Fig. 12A). MS^2 of the ion at *m/z* 948 identified a ganglioside with NeuAc-Hex-HexNAc-Hex-HexNAc-Hex-Hex sequence, and with d18:1-h16:0 ceramide, while MS^2 of the ion at *m/z* 912 identified GD1b ganglioside with d18:1-h16:0 ceramide, as described for fraction IV above (data not shown).

The $[M\text{-}2H^+]^{2-}$ ion at *m/z* 1123 corresponded to a $[M\text{-}H^+]^-$ ion at *m/z* 2247, and indicated a ganglioside with one NeuAc, three HexNAc, five Hex, and d18:1-16:0 ceramide. The MS^2

spectrum (Fig. 12B) had a Y ion series (Y_0 at *m/z* 536, Y_1 at *m/z* 698, Y_2 at *m/z* 860, Y_5 at *m/z* 1428, Y_6 at *m/z* 1590, and Y_8 at *m/z* 1956), which together with the B and C type ions gave identification of a NeuAc-Hex-HexNAc-Hex-HexNAc-Hex-HexNAc-Hex-Hex ganglioside with d18:1-16:0 ceramide.

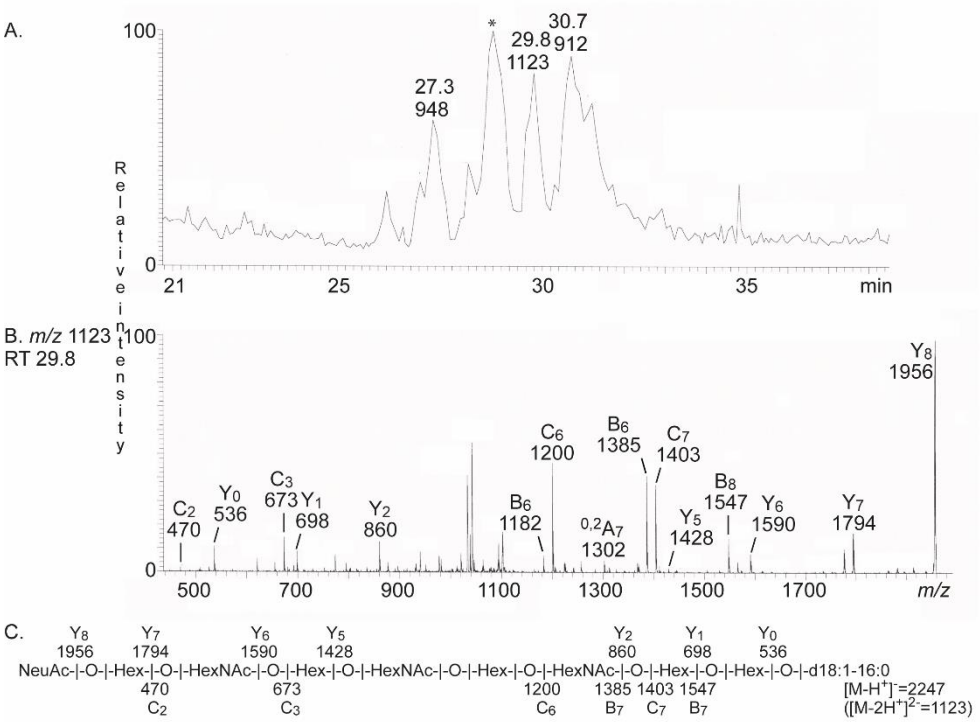

Fig. 12. LC-ESI/MS of the acid glycosphingolipid fraction V isolated from a human lung cancer metastasis.

A. Base peak chromatogram. The * marks a non-glycosphingolipid contaminant.

B. MS^2 spectrum of the $[M-2H^+]^{2-}$ ion at *m/z* 1123 (retention time 29.8 min).

C. Interpretation formula showing the deduced glycosphingolipid sequence.

Chromatogram binding assays

In order to extend the structural information obtained by mass spectrometry, binding of a number of carbohydrate binding ligands to the acid glycosphingolipid fractions of the lung cancer metastasis was next examined in a chromatogram binding assay. The fucose-recognizing *A. aurantia* lectin [39] bound to slow-migrating compounds in fractions III-IV (Fig. 13B**,** lanes 4-6), indicating the presence of complex fucosylated gangliosides in these fractions. There was also a distinct interaction of the anti-NeuAcα3-neolacto antibodies to all isolated fractions used (Fig. 13C, lanes 2-6), demonstrating that all these fractions contained gangliosides with terminal NeuAcα3-neolacto (NeuAcα3Galβ4GlcNAc) sequences. The anti-NeuAcα3-lacto antibodies on the other hand bound only to

a compound in fraction II (Fig. 13D, lane 3), along with binding to the total acid fraction of human lung cancer metastasis (lane 1). This binding indicated that fraction II contained a ganglioside with terminal NeuAcα3-lacto (NeuAcα3Galβ3GlcNAc) sequence.

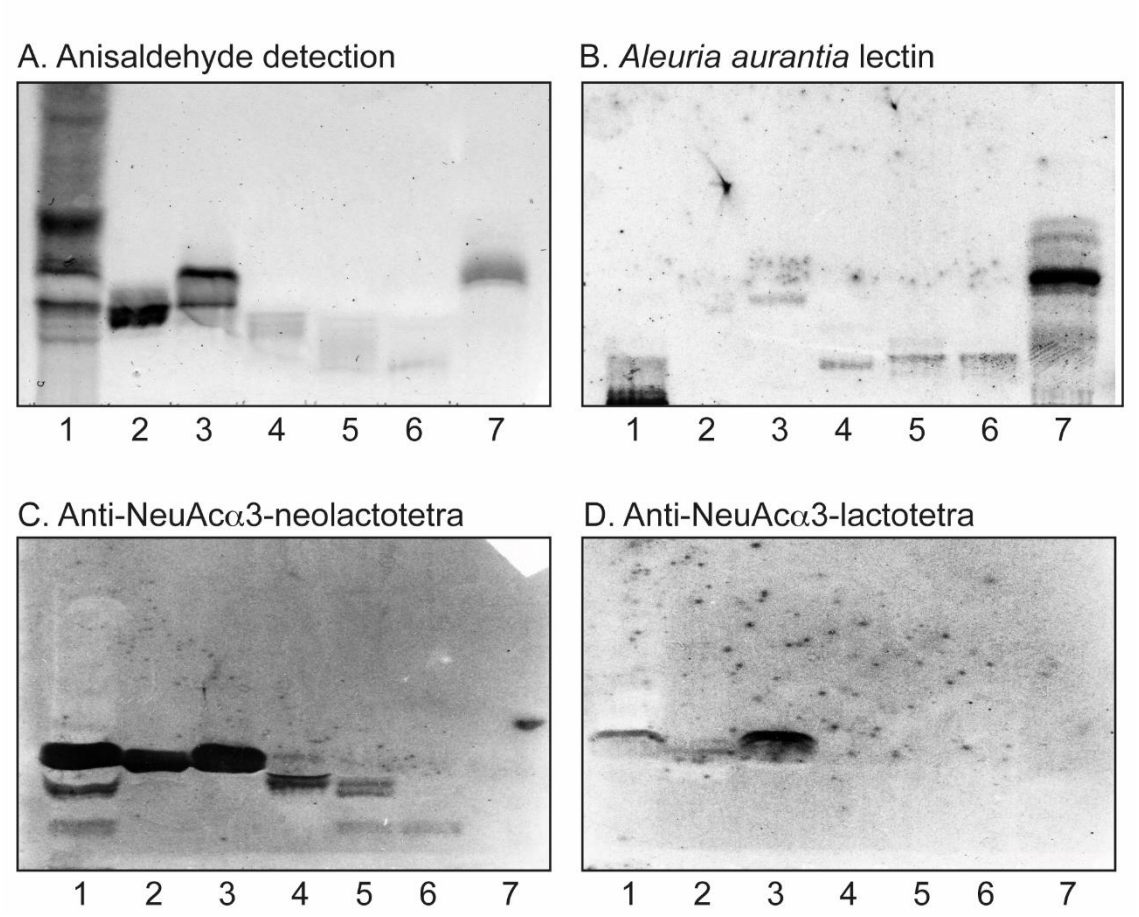

Fig. 13. Characterization of the acid glycosphingolipid fractions isolated from human lung cancer metastasis by antibody and lectin binding. Thin-layer chromatogram after detection with anisaldehyde (A), and autoradiograms obtained by binding of *A. aurantia* lectin (B), the anti-NeuAcα3-neolactotetra antibody (C), and the anti-NeuAcα3-lactotetra antibody (D). The lanes were: Lane 1, total acid glycosphingolipids of human lung cancer metastasis, 40 μg; Lane 2, fraction I isolated from human lung cancer metastasis, 4 μg; Lane 3, fraction II, 4 μg; Lane 4, fraction III, 1 μg; Lane 5, fraction IV, 1 μg; Lane 6, fraction V, 1 μg; Lane 7, reference Le^b hexaosylceramide, 4 μg.

Table 3. Ganglioside characterization. Summary of results from LC-ESI/MS and chromatogram binding assays.

	Sequence from LC-ESI/MS	αnL[1]	αL[2]	AAL[3]	Ganglioside structure/Trivial name
I	NeuAcα3Hex-HexNAc-Hex-Hex-d18:1-16:0 NeuAcα6Hex-HexNAc-Hex-Hex-d18:1-16:0	+[4]	-	-	NeuAcα3Galβ4GlcNAcβ3Galβ4Glc = **NeuAcα3SPG** NeuAcα6Galβ4GlcNAcβ3Galβ4Glc = **NeuAcα6SPG**
II	NeuAcα3Hex-HexNAc-Hex-Hex-d18:1-16:0 NeuAcα3Hex-HexNAc-Hex-Hex-d18:1-24:0	+	+	+	NeuAcα3Galβ4GlcNAcβ3Galβ4Glc = **NeuAcα3SPG** NeuAcα3Galβ3GlcNAcβ3Galβ4Glc = **NeuAcα3-SL4**
III	NeuAc-Hex-HexNAc-Hex-HexNAc-Hex-Hex-d18:1-16:0 Hex-HexNAc-(NeuAc-NeuAc-)Hex-Hex-d18:1-16:0	+	-	+	NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc = **NeuAcα3nLc6** Galβ3GalNAcβ4(NeuAcα8NeuAcα3)Galβ4Glc = **GD1b**
IV	NeuAc-Hex-HexNAc-Hex-HexNAc-Hex-Hex-d18:1-h16:0 NeuAc-Hex-HexNAc-Hex-(Fuc-)HexNAc-Hex-Hex-d18:1-h16:0 Hex-HexNAc-(NeuAc-NeuAc-)Hex-Hex-d18:1-h16:0	+	-	+	NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc = **NeuAcα3nLc6** NeuAcα3Galβ4GlcNAcβ3Galβ4(Fucα3)GlcNAcβ3Galβ4Glc = **VIM2** Galβ3GalNAcβ4(NeuAcα8NeuAcα3)Galβ4Glc = **GD1b**
V	NeuAc-Hex-HexNAc-Hex-HexNAc-Hex-Hex-d18:1-h16:0 Hex-HexNAc-(NeuAc-NeuAc-)Hex-Hex-d18:1-h16:0 NeuAc-Hex-HexNAc-Hex-HexNAc-Hex-HexNAc-Hex-Hex-d18:1-16:0	+	-	+	NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc = **NeuAcα3nLc6** Galβ3GalNAcβ4(NeuAcα8NeuAcα3)Galβ4Glc = **GD1b** NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc = **NeuAcα3nLc8**

[1]αnL monoclonal antibodies directed against the NeuAcα3-neolacto epitope (clone LM1:1a).

[2]αL, monoclonal antibodies directed against the NeuAcα3-lacto epitope (clone TR4/SL-50).

[3]AAL, *Aleuria aurantia* lectin.

[4]Binding is defined as follows: + denotes a staining when 4 μg of the glycosphingolipid fraction was applied on the thin-layer chromatogram, while - denotes no binding even at 4 μg.

H. pylori SabA binding

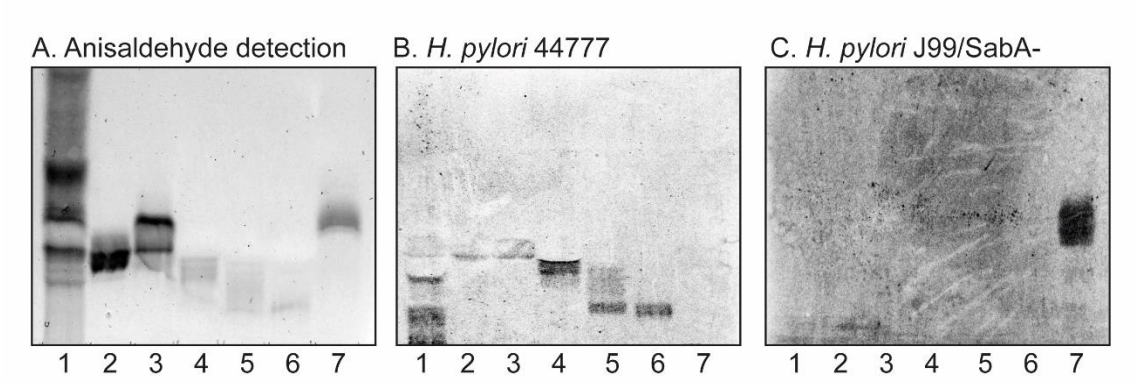

Fig. 14. Binding of SabA expressing *H. pylori* strain 44777 and the SabA knock-out strain J99/SabA- to the acid glycosphingolipid fractions isolated from human lung cancer metastasis. Thin-layer chromatogram after detection with anisaldehyde (A), and autoradiograms obtained by binding ^{35}S-labeled *H. pylori* strain 44777 (B), and *H. pylori* strain J99/SabA- (C). The lanes were: Lane 1, total acid glycosphingolipids of human lung cancer metastasis, 40 μg; Lane 2, fraction I isolated from human lung cancer metastasis, 4 μg; Lane 3, fraction II, 4 μg; Lane 4, fraction III, 1 μg; Lane 5, fraction IV, 1 μg; Lane 6, fraction V, 1 μg; Lane 7, reference Le^b hexaosylceramide, 4 μg.

Table 4. Summary of results from binding of SabA-expressing *H. pylori* on thin-layer chromatograms

	Ganglioside structure/Trivial name	**SabA binding[1]**
I	NeuAcα3Galβ4GlcNAcβ3Galβ4Glc = **NeuAcα3SPG** NeuAcα6Galβ4GlcNAcβ3Galβ4Glc = **NeuAcα6SPG**	-
II	NeuAcα3Galβ4GlcNAcβ3Galβ4Glc = **NeuAcα3SPG** NeuAcα3Galβ3GlcNAcβ3Galβ4Glc = **NeuAcα3-SL4**	-
III	NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc = **NeuAcα3nLc6** Galβ3GalNAcβ4(NeuAcα8NeuAcα3)Galβ4Glc = **GD1b**	+
IV	NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc = **NeuAcα3nLc6** NeuAcα3Galβ4GlcNAcβ3Galβ4(Fucα3)GlcNAcβ3Galβ4Glc = **VIM2** Galβ3GalNAcβ4(NeuAcα8NeuAcα3)Galβ4Glc = **GD1b**	+
V	NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc = **NeuAcα3nLc6** Galβ3GalNAcβ4(NeuAcα8NeuAcα3)Galβ4Glc = **GD1b** NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc = **NeuAcα3nLc8**	+

[1]Binding is defined as follows: + denotes an intense and highly reproducible staining when 4 μg of the glycosphingolipid was applied on the thin-layer chromatogram, while - denotes no binding even at 4 μg.

7. DISCUSSION

Glycosylation changes have been known as characteristic feature in cancer cells and alterations in sialic acid and fucose expression is one part of that complex process. It usually includes higher representation of sialic acid on the cell surface. Moreover, it has been demonstrated that fucosylated glycoconjugates as Lewis^a and Lewis^x are generally overexpressed in cancerogenesis [1].

In the initial phase of the project, a number of acid glycosphingolipid fractions from various sources were tested for binding of SabA-expressing *H. pylori* using the chromatogram binding assay. Thereby, binding of the bacteria to slow-migrating complex gangliosides in the acid glycosphingolipid fraction of a human liver metastasis from lung cancer was obtained (Fig. 15, lane 2), along with previously described binding to acid glycosphingolipids of human eosinophils (lane 3) and NeuAcα3-neolactotetraosylceramide (lane 4).

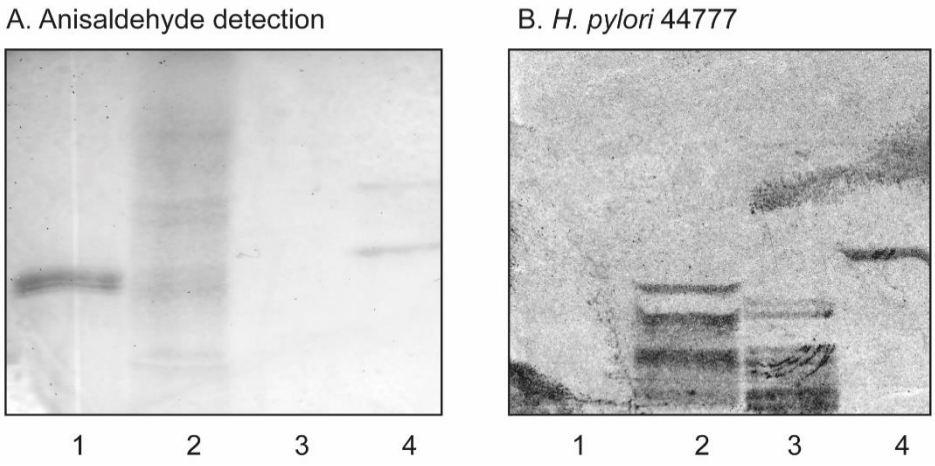

Fig. 15. Binding of *H. pylori* to glycosphingolipids on thin-layer chromatograms.
The chromatogram in (A) was stained with anisaldehyde. A duplicate chromatogram was incubated with ^{35}S-labeled *H. pylori* strain 44777, followed by autoradiography for 12 h. The lanes were: Lane 1, reference Le^b hexaosylceramide, 4 μg; Lane 2, acid glycosphingolipids of a human lung cancer metastasis, 40 μg; Lane 3, acid glycosphingolipids of human eosinophils, 40 μg; Lane 4, reference NeuAcα3-neolactotetraosylceramide, 4 μg.

Thus, in this thesis, a sample of human liver metastasis from lung cancer was used as a source of potential *H. pylori*-binding gangliosides. First of all, column chromatography using different types of silica gel as stationary phase was performed to purify and isolate the

gangliosides from the total fraction. The presence of halogen salts in the mobile phase has a noticeable influence on the mobility, because the strongly ionized cations are supposed to associate with gangliosides thereby leads to better separation of that GSLs-ion complexes on silica gel [40].

For closer characterization, a series of chromatogram binding assays were executed. This technique is based on the specific binding of ligands to the separated GSLs and it represents an easy and fast way to localize the biologically active compound on the silica plate. Various compound can be used as ligands such as bacteria, antibodies, lectins as well as carbohydrate-specific viruses and bacterial toxins. Furthermore, the overlay technique is also valuable in finding and recognizing GSLs structures as potential receptors for viruses, bacteria and cells. Due to the high sensitivity of this method, even nanogram amounts of GSLs could be enough for satisfying results [41]

In summary, based on those results combined with HILIC-ESI-MS structure analysis, we obtained and characterized three different *H. pylori* SabA binding gangliosides, including NeuAcα3Galβ4GlcNAcβ3Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer (=VIM2 ganglioside), NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer (=NeuAcα3neolactohexaosyl-ceramide) and with longer core chain NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3 Galβ4GlcNAcβ3Galβ4Glcβ1Cer (=NeuAcα3-neolactooctaosyl-ceramide). All these gangliosides had been recognized as *H. pylori* active-binding ones before and their degree of affinity were demonstrated as well. It had been observed that increased length of *N*-acetyllactosamine core chain improves the availability of head group and thus enhances the binding affinity of *H. pylori.* Furthermore, the fucose substitution also plays the role, as it provides stronger interactions with SabA binding site and influences the ganglioside conformation, thereby delivers optimal offering of epitope [42].

All the techniques and appropriate conditions were chosen and performed based on the previous studies and experience of the research group.

Despite strenuous efforts we were not able to obtain the *H. pylori* binding gangliosides as pure compounds. Thus, deductions about binding and non-binding compounds were done using ganglioside mixtures containing VIM2 ganglioside, NeuAcα3-neolactohexaosylceramide and NeuAcα3-neolactooctaosylceramide along GD1b.

Further purifications and structural characterization, by *e.g*. H-NMR, will be done to fully establish the structures of the *H. pylori* SabA binding gangliosides.

The crystal structure of the extracellular adhesion domain of *H. pylori* sialic binding adhesin SabA has been solved to 2.2 Å. The studies revealed a predominantly α-helical molecule with one “handle” and one “head” region, thus an overall shape is similar to a golf

putter [43]. The same handle-head architecture is found in the crystal structure of the extracellular adhesion domain of *H. pylori* Le^b-binding adhesin BabA. In addition, the BabA adhesin has a ”crown” region harbouring the Le^b binding site [44]. Compared to that, the sialic acid binding site of SabA has not been identified yet, although a putative glycan binding pocket was suggested by point mutation studies. However, attempts to co-crystalize SabA with sialyl-Le^x failed. Thus, identification of SabA ligands that are recognized with higher affinity are needed to get crystallographic insight into the structural basis of SabA recognition of sialic acid carrying glycans.

Importance

As it has been mentioned, infection inducing by *H. pylori* entails much more problems than only gastric mucosal inflammation. It correlates with increased risk of gastric cancer development and is reasonably classified as carcinogen class I. Despite a great progression in both medicinal and scienficic field over the years, the effective vaccine has not been discovered yet. Coupled with an enhancement in antibiotics resistence, which represent an essential part of infection treatment, there are still a lot of issues to study and improve.

Therefore, novel and improved schemes for treating *H. pylori* infection are urgently needed, and *e.g*. development of therapeutics targeting *H. pylori* virulence factors represents one of the promising strategies in the future.

One such alternative therapy using carbohydrate receptor analogues as antiadhesion compounds for inhibition of the initial bacterial attachment to the gastric epithelium. Preliminary studies demonstrating sialyl-lactose to suppress *H. pylori* colonization gave no provable effect. The results indicated the necessity to use ligands with more elevated affinity to SabA. For successful structure-based design of oligosaccharide analogues as high affinity receptor, the knowledge of SabA carbohydrate binding sites at an atomic level is an indispensable basis [45].

Weaknesses

Initially, we intended to use the *H. pylori* strain J99 and the J99/SabA knockout strain in parallel, for the reason that J99 strain has both BabA and SabA and thus binds to either Le^b- and sialic acid-carrying glycoconjugates. However, despite repeated attempts, we were not able to get a ganglioside binding with the J99 strain, therefore the *H. pylori* strain 44777 was used instead. Nevertheless, phase variation of SabA has previously been reported, and several mechanisms for regulation of protein expression have been proposed as well, as *e.g*. translational frameshifts caused by a cytosine-thymine dinucleotide repeat tract in the

5’-end of the *sabA* coding sequence [36]. More recently it has been shown that the length of a thymine nucleotide repeat tract adjacent to the *sabA*-35 promoter element controls the *sabA* transcription initiation by affecting the binding of the RNA polymerase [46].

8. CONCLUSION

The main aim of the diploma thesis was the recognition and structural characterization of SabA binding gangliosides isolated from the total acid fraction of human lung cancer metastasis. In summary, we obtained three different gangliosides that were bound to *H. pylori* strain expressing SabA, including NeuAcα3Galβ4GlcNAcβ3Galβ4 (Fucα3)GlcNAcβ3Galβ4Glcβ1Cer (=VIM2 ganglioside), NeuAcα3Galβ4GlcNAcβ3Galβ4 GlcNAcβ3Galβ4Glcβ1Cer (=NeuAcα3-neolactohexaosylceramide) and NeuAcα3Galβ4 GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer (=NeuAcα3neolactooctaosyl-ceramide). Despite strenuous efforts, it was not able to obtain the gangliosides as pure compounds. Thus, further purifications and structural characterization, by *e.g*. proton NMR, will be performed in the near future to fully establish the structures. That results could be subsequently used for the design and development of novel scheme in *H. pylori* infection treatment, focusing on inhibition of the initial bacterial attachment to the gastric epithelium. This alternative therapy using carbohydrate receptor analogues as antiadhesion compounds definitely represents one of the promising strategies in this field.

9. REFERENCES

1. VARKI, A.; CUMMINGS, RD.; ESKO, JD, et al., editors. *Essentials of Glycobiology*. 2nd edition. New York: Cold Spring Harbour Laboratory Press, 2009. 784 pp. ISBN: 9780879697709.
2. KONTUREK, JW. Discovery by Jaworski of Helicobacter pylori. *J. Physiol. Pharmacol.* 2003, 54, 23-41. ISSN: 0867-5910.
3. KUSTERS, JG.; VAN VLIET, AHM; KUIPERS, EJ. Pathogenesis of Helicobacter pylori infection. *Clin. Microbiol. Rev.* 2006, 19, 449-490. ISSN: 0893-8512.
4. OWEN, RJ. Helicobacter-species classification and identification. *Br. Med. Bull.* 1998, 54, 17-30. ISSN: 0007-1420.
5. SUERBAUM, S.; MICHETTI, P. Helicobacter pylori infection. *N. Engl. J. Med.* 2002, 347, 1175-1186. ISSN: 0028-4793.
6. DE FALCO, M.; LUCARIELLO, A.; IAQUINTO, S.; ESPOSITO, V.; GUERRA, G.; DE LUCA, A. Molecular mechanisms of Helicobacter pylori pathogenesis. *J. Cell. Physiol.* 2015, 230, 1702-1707. ISSN: 0021-9541.
7. MARTÍNEZ, LE.; HARDCASTLE, JM.; WANG, J.; PINCUS, Z.; TSANG, J.; HOOVER, TR.; BANSIL, R.; SALAMA, NR. Helicobacter pylori strains vary cell shape and flagellum number to maintain robust motility in viscous environments. *Mol. Microbiol.* 2016, 99, 88-110. ISSN: 0950-382X.
8. SGOURAS, DN.; TRANG, TTH; YAMAOKA, Y. Pathogenesis of Helicobacter pylori Infection. *Helicobacter*. 2015, 20, 8-16. ISSN: 1083-4389.
9. SPOHN, G.; SCARLATO, V. Motility, chemotaxis, and flagella. In: MOBLEY, HLT., et al. *Helicobacter pylori: Physiology and Genetics*. Washington (DC): ASM Press, 2001. 608 pp. ISBN: 9781555812133.
10. SCHREIBER, S.; KONRADT, M.; GROLL, C.; SCHEID, P.; HANAUER, G.; WERLING, HO.; JOSENHANS, C.; SUERBAUM, S. The spatial orientation of Helicobacter pylori in the gastric mucus. *Proc. Natl. Acad. Sci. U. S. A.* 2004, 101, 5024-5029. ISSN: 0027-8424.
11. MAGALHAES, A.; REIS, CA. Helicobacter pylori adhesion to gastric epithelial cells is mediated by glycan receptors. *Brazilian J. Med. Biol. Res.* 2010, 43, 611-618. ISSN: 0100-879X.
12. SATIN, B.; DEL GIUDICE, G.; DELLA BIANCA, V.; DUSI, S.; LAUDANNA, C.; TONELLO, F.; KELLEHER, D.; RAPPUOLI, R.; MONTECUCCO, C.; ROSSI, F. The neutrophil-activating protein (HP-NAP) of Helicobacter pylori is a protective antigen and a major virulence factor. *J. Exp. Med.* 2000, 191, 1467-1476. ISSN: 0022-1007.
13. ATKINSON, NSS; BRADEN, B. Helicobacter Pylori Infection: Diagnostic Strategies in Primary Diagnosis and After Therapy. *Dig. Dis. Sci.* 2015, 61, 1-6. ISSN: 0163-2116.
14. GARZA-GONZÁLEZ, E.; PEREZ-PEREZ, GI.; MALDONADO-GARZA, HJ.; BOSQUES-PADILLA, FJ. A review of Helicobacter pylori diagnosis, treatment, and methods to detect eradication. *World J. Gastroenterol.* 2014, 20, 1438-1449. ISSN: 1007-9327.
15. MENTIS, A.; LEHOURS, P.; MÉGRAUD, F. Epidemiology and Diagnosis of Helicobacter pylori infection. *Helicobacter*. 2015, 20, 1-7. ISSN: 1083-4389.
16. SIPPONEN, P.; HYVÄRINEN, H. Role of Helicobacter pylori in the pathogenesis of gastritis, peptic ulcer and gastric cancer. *Scand. J. Gastroenterol.* 1993, 28, 3-6. ISSN: 0036-5521.

17. MCCOLL, KEL. Helicobacter pylori infection. *N. Engl. J. Med.* 2010, 362, 1597-1604. ISSN: 0028-4793.
18. KHAN, MQ. Helicobacter pylori eradication therapy in nonulcer dyspepsia is beneficial. *Saudi J. Gastroenterol.* 2008, 14, 96. ISSN: 1319-3767.
19. ISLAMI, F.; KAMANGAR, F. Helicobacter pylori and esophageal cancer risk: a meta-analysis. *Cancer Prev. Res.* 2008, 1, 329-338. ISSN: 1940-6207.
20. NIE, S.; CHEN, T.; YANG, X.; HUAI, P.; LU, M. Association of Helicobacter pylori infection with esophageal adenocarcinoma and squamous cell carcinoma: a meta-analysis. *Dis. Esophagus*. 2014, 27, 645-653. ISSN: 1120-8694.
21. STOLZENBERG-SOLOMON, RZ.; BLASER, MJ.; LIMBURG, PJ.; PEREZ-PEREZ, G.; TAYLOR, PR.; VIRTAMO, J.; ALBANES, D. Helicobacter pylori seropositivity as a risk factor for pancreatic cancer*. J. Natl. Cancer Inst.* 2001, 93, 937-941. ISSN: 0027-8874.
22. SCHULTE, A.; PANDEYA, N.; FAWCETT, J.; FRITSCHI, L.; RISCH, HA.; WEBB, PM.; WHITEMAN, DC.; NEALE, RE. Association between Helicobacter pylori and pancreatic cancer risk: a meta-analysis. *Cancer Causes Control*, 2015, 26, 1-9. ISSN: 0957-5243.
23. SHMUELY, H.; PASSARO, D.; FIGER, A.; NIV, Y.; PITLIK, S.; SAMRA, Z.; KOREN, R.; YAHAV, J. Relationship between Helicobacter pylori CagA status and colorectal cancer. *Am. J. Gastroenterol.* 2001, 96, 3406-3410. ISSN: 0002-9270.
24. SELGRAD, M.; BORNSCHEIN, J.; KANDULSKI, A.; HILLE, C.; WEIGT, J.; ROESSNER, A.; WEX, T.; MALFERTHEINER, P. Helicobacter pylori but not gastrin is associated with the development of colonic neoplasms. *Int. J. Cancer.* 2014, 135, 1127-1131. ISSN: 0020-7136.
25. VENERITO, M.; VASAPOLLI, R.; ROKKAS, T.; MALFERTHEINER, P. Helicobacter pylori and Gastrointestinal Malignancies. *Helicobacter*, 2015, 20, 36-39. ISSN: 1083-4389.
26. LEE, JW.; LEE, DH.; LEE, JI.; JEONG, S.; KWON, KS.; KIM, HG.; SHIN, YW.; KIM, YS.; CHOI, MS.; SONG, SY. Identification of Helicobacter pylori in gallstone, bile, and other hepatobiliary tissues of patients with cholecystitis. *Gut Liver*, 2010, 4, 60-67. ISSN: 1976-2283.
27. WALUGA, M.; KUKLA, M.; ŻORNIAK, M.; BACIK, A.; KOTULSKI, R. From the stomach to other organs: Helicobacter pylori and the liver. *World J. Hepatol*. 2015, 7, 2136. ISSN: 1948-5182.
28. FRANCESCHI, F.; TORTORA, A.; GASBARRINI, G.; GASBARRINI, A. Helicobacter pylori and extragastric diseases. *Helicobacter*, 2014, 19, 52-58. ISSN: 1083-4389.
29. FRANCESCHI, F.; GASBARRINI, A.; STERGIOS, AP.; KOUNTOURA, J. Extragastric Diseases and Helicobacter pylori. *Helicobacter*, 2015, 20, 40-46. ISSN: 1083-4389.
30. JORK, H.; FUNK, W.; FISCHER, W.; WIMMER, H. *Thin-Layer Chromatography: Reagents and Detection Methods*. Weinheim: VCH, 1990. 464 pp. ISBN 3-527-27834-6.
31. MÜTHING, J. High-resolution thin-layer chromatography of gangliosides. *J. Chromatogr. A.* 1996, 720, 3-25. ISSN: 0021-9673.
32. SVENNERHOLM, L. Quantitive estimation of sialic acids: II. A colorimetric resorcinol-hydrochloric acid method. *Biochim. Biophys. Acta*. 1957, 24, 604-611. ISSN: 1388-1981.
33. ROCHE, N.; LARSSON, T.; ÅNGSTRÖM, J.;TENEBERG, S. Helicobacter pylori–binding gangliosides of human gastric adenocarcinoma. *Glycobiology*. 2001, 11, 935-944. ISSN: 0959-6658.

34. SVENNERHOLM, L.; BOSTRÖM, K.; FREDMAN, P.; MÅNSSON, JE.; ROSENGREN, B.; RYNMARK, B. M. Human brain gangliosides: developmental changes from early fetal stage to advanced age. *Biochim. Biophys. Acta*. 1989, 1005, 109-117. ISSN: 1388-1981.
35. SVENNERHOLM, L.; RYNMARK, BM.; VILBERGSSON, G.; FREDMAN, P.; GOTTFRIES, J.; MÅNSSON, JE.; PERCY, A. Gangliosides in human fetal brain. *J. Neurochem.* 1991, 56, 1763-1768. ISSN: 0022-3042.
36. MAHDAVI, J.; SONDÉN, B.; HURTIG, M.; OLFAT, FO.; FORSBERG, L.; ROCHE, N.; ÅNGSTRÖM, J.; LARSSON, T.; TENEBERG, S.; KARLSSON, KA.; ALTRAJA, S.; WADSTRÖM, T.; KERSULYTE, D.; BERG, DE.; DUBOIS, A.; PETERSSON, C.; MAGNUSSON, KE.; NORBERG, T.; LINDH, F.; LUNDSKOG, BB.; ARNQVIST, A.; HAMMARSTRÖM, L.; BOREN, T. Helicobacter pylori SabA adhesin in persistent infection and chronic inflammation. *Science*. 2002, 297, 573-578. ISSN: 0036-8075.
37. MEISEN, I.; PETER-KATALINIC, J.; MÜTHING, J. Discrimination of neolacto-series gangliosides with α2-3-and α2-6-linked N-acetylneuraminic acid by nanoelectrospray ionization low-energy collision-induced dissociation tandem quadrupole TOF MS. *Anal. Chem.* 2003, 75, 5719-5725. ISSN: 0003-2700.
38. ITO, E.; TOMINAGA, A.; WAKI, H.; MISEKI, K.; TOMIOKA, A.; NAKAJIMA, K.; KAKEHI, K.; SUZUKI, M.; TANIGUCHI, N.; SUZUKI, A. Structural characterization of monosialo-, disialo-and trisialo-gangliosides by negative ion AP-MALDI-QIT-TOF mass spectrometry with MSn switching. *Neurochem. Res.* 2012, 37, 1315-1324. ISSN: 0364-3190.
39. KOCHIBE, N.; FURUKAWA, K. Purification and properties of a novel fucose-specific hemagglutinin of Aleuria aurantia. *Biochemistry*. 1980, 19, 2841-2846. ISSN: 0006-2960.
40. MUTHING, J. TLC in structure and recognition studies of glycosphingolipids. In: HOUNSELL, EF., editor. *Glycoanalysis Protocols*. New York: Humana Press, 1998. p. 183-195. ISBN 978-1-59259-562-4.
41. MEISEN, I.; MORMANN, M.; MÜTHING, J. Thin-layer chromatography, overlay technique and mass spectrometry: a versatile triad advancing glycosphingolipidomics. *Biochim. Biophys. Acta*. 2011, 1811, 875-896. ISSN: 1388-1981.
42. ROCHE, N.; ÅNGSTRÖM, J.; HURTIG, M.; LARSSON, T.; BORÉN, T.; TENEBERG, S. Helicobacter pylori and complex gangliosides. *Infect. Immun*. 2004, 72, 1519-1529. ISSN: 0019-9567.
43. PANG, SS.; NGUYEN, STS.; PERRY, AJ.; DAY, CJ.; PANJIKAR, S.; TIRALONGO, J.; WHISSTOCK, JC.; KWOK, T. The three-dimensional structure of the extracellular adhesion domain of the sialic acid-binding adhesin SabA from Helicobacter pylori. *J. Biol. Chem.* 2014, 289, 6332-6340. ISSN: 0021-9258.
44. HAGE, N.; HOWARD, T.; PHILLIPS, C.; BRASSINGTON, C.; OVERMAN, R.; DEBRECZENI, J.; GELLERT, P.; STOLNIK, S.; WINKLER, GS.; FALCONE, FH. Structural basis of Lewisb antigen binding by the Helicobacter pylori adhesin BabA. *Sci. Adv.* 2015, 1, e1500315. ISSN 2375-2548.
45. PARENTE, F.; CUCINO, C.; ANDERLONI, A.; GRANDINETTI, G.; PORRO, GB. Treatment of Helicobacter Pylori Infection Using a Novel Antiadhesion Compound (3′ sialyllactose sodium salt). A Double blind, Placebo-Controlled Clinical Study. *Helicobacter*. 2003, 8, 252-256. ISSN: 1083-4389.

46. ÅBERG, A.; GIDEONSSON, P.; VALLSTRÖM, A.; OLOFSSON, A.; ÖHMAN, C.; RAKHIMOVA, L.; BORÉN, T.; ENGSTRAND, L.; BRÄNNSTRÖM, K.; ARNQVIST, A. A repetitive DNA element regulates expression of the Helicobacter pylori sialic acid binding adhesin by a rheostat-like mechanism. *PLoS Pathog*. 2014, 10, e1004234. ISSN: 1553-7366.

FIGURES

Fig. 2.: Shutterstock-helicobacter-pylori. In: SteadyHealt [online]. SteadyHealth.com © 2015. [vid 25.11.2015]. From: http://userfiles.steadyhealth.com/userfiles/articles/shutterstock-helicobacter-pylori.jpg

Fig. 3.: H_pylori_virulence_factors_en. In: Wikimedia Commons [online]. This file is licensed under the terms of the GNU Free Documentation License. [vid 30.11.2015]. From: https://commons.wikimedia.org/wiki/File:H_pylori_virulence_factors_en.png

Fig. 8.: Types of carbohydrate fragmentation. In: A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates. [online]. DOMON, Bruno; COSTELLO, Catherine E. 1988 [vid 10.02.2016]. From: http://link.springer.com/article/10.1007/BF01049915